AD_____

AWARD NUMBER: W81XWH-07-1-0531

TITLE: Discovery and Role of a Novel Eicosanoid Family in Breast Cancer

PRINCIPAL INVESTIGATOR: Claus Schneider, Ph.D.

CONTRACTING ORGANIZATION: Vanderbilt University Medical Center
Nashville, TN 37203

REPORT DATE: August 2009

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
1 August 2009	Final	1 Aug 2007 – 31 Jul 2009

4. TITLE AND SUBTITLE

Discovery and Role of a Novel Eicosanoid Family in Breast Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-07-1-0531

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Claus Schneider, Ph.D.

E-Mail: claus.schneider@vanderbilt.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Vanderbilt University Medical Center
Nashville, TN 37203

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
There is strong evidence for the induction of the pro-inflammatory enzymes 5-lipoxygenase (5-LOX) and cyclooxygenase-2 (COX-2) in many types of cancer, including breast cancer. Up regulation of both enzymes is associated with promoting tumor igenesis and a negative prognosis of the disease. The 5-LOX (leukotriene) and COX-2 (prostaglandin) pathways have traditionally been viewed as independent biosynthetic routes to eicosanoid lipid hormones, yet recent enzy-mological analyses establish that the 5-LOX product, 5-HETE, is an excellent COX-2 substrate, forming a novel double endoperoxide product that has the potential to evolve into a novel family of biomediators. We have used LC-MS and NMR to identify two cellular metabolites that are derived from the novel di-endoperoxide product. Formation of the metabolites is blocked by presence of the COX-2 inhibitor indomethacin. Detection of these products is a major step forward in establishing a potential novel eicosanoid-forming pathway resulting from the cross-over of the leukotriene and prostaglandin pathways in breast cancer.

15. SUBJECT TERMS
Eicosanoid, COX-2, 5-lipoxygenase, arachidonic acid

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	70	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Page

Introduction

There is strong evidence for the induction of the pro-inflammatory enzymes 5-lipoxygenase (5-LOX) and cyclooxygenase-2 (COX-2) in many types of cancer, including breast cancer. Upregulation of either enzyme is associated with promoting tumorigenesis and a negative prognosis of the disease. The 5-LOX (leukotriene) and COX-2 (prostaglandin) pathways have traditionally been viewed as independent biosynthetic routes to eicosanoid lipid hormones because either enzyme catalyzes the initiating transformation of arachidonic acid toward their respective pathway. Yet our recent enzymological analyses have established that the 5-LOX product, 5*S*-HETE, is an excellent substrate for COX-2, forming a novel di-endoperoxide [1]. Similar to the transformation of the prostaglandin endoperoxide PGH_2, the 5-HETE-derived di-endoperoxide has the potential to evolve into a family of highly oxygenated eicosanoid biomediators. In this project we tested the hypothesis that novel eicosanoids derived from the cross-over of the 5-LOX and COX-2 pathways can be detected in breast cancer cells that are positive for both enzymes. The long-term goal of this project is to investigate how these novel eicosanoids regulate proliferation and differentiation of breast cancer cells, and thus, to elucidate whether pharmacological manipulation of formation of these novel eicosanoids can provide a strategy for the treatment of breast cancer.

Body

Task 1. <u>To investigate the expression of 5-LOX and COX-2 in breast cancer cell lines</u>

The commercially available antibodies for 5-LOX give inconsistent and partially false positive signals (Fig. 1). We therefore decided to generate our own peptide-derived antibody for 5-LOX through a commercial outside contractor. The new polyclonal antibody is quite sensitive and highly specific with no cross-reaction to the other five LOX isoforms. We have screened a range of human breast cancer cell lines for expression of 5-LOX and COX-2 using Western blotting.

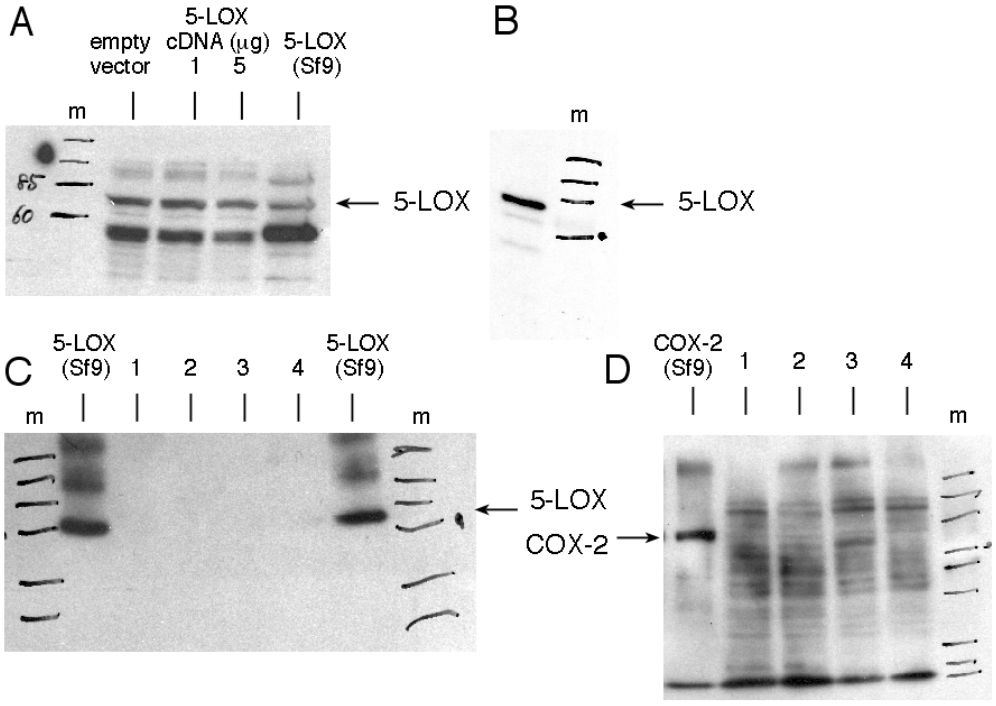


Fig. 1. Western blot detection of 5-LOX and COX-2. (A) An antibody for 5-LOX obtained from BD Biosciences detects a band that co-migrates with 5-LOX in COS-7 cells transfected with empty vector (negative control!), with 5-LOX cDNA at 1 and 5 µg, and 5-LOX expressed in Sf9 insect cells. (B) We have generated a peptide antibody that is specific for detection of human 5-LOX in Western blotting. (C) Using the antibody shown in (B) we did not detect expression of 5-LOX in H2122 (lane 1), MCF-7 (lane 2), MDA-MB-231 (lane 3), or HCA7 cells (lane 4). (D) Expression of COX-2 was not detected in H2122, MCF-7, MDA-MB-231, or HCA7 cells (same loading order as in (C)).

We have not succeeded in detecting expression of either 5-LOX or COX-2 in the breast cancer (MCF-7, MDA-MB-231) or other cancer cell lines (H2122, HCA7) that we have tested.

Task 2. <u>To investigate the formation of novel 5-LOX/COX-2 dependent metabolites in breast cancer cell lines</u>

We investigated the transformation of 5-HETE in our current model of RAW264.7 macrophages that express high levels of COX-2 upon activation with LPS/IFN-γ. We prepared 5*S*-HETE substrate labeled with deuterium at carbons 5,6,8,9,11,12,14,15 starting from d_8-arachidonic acid and incubation with recombinant 5-lipoxygenase expressed in *Sf9* insect cells.

Using d_8-labeled 5*S*-HETE **1** we observed formation of the previously identified di-endoperoxide **2** and two novel eicosanoids, **3** and **4**, when using recombinant human COX-2 (Fig. 2, top panel). Incubation of 5*S*-HETE with activated RAW264.7 cells gave the same three products, albeit the ratio of products **3** to **4** was changed such that **4** was the more prominent product in the cellular incubations (Fig. 2, middle panel). The isotopic pattern clearly indicated that these products were derived from 5*S*-HETE, but whereas the di-endoperoxide **2** retained all 8 deuterium labels, the novel metabolites **3** and **4** retained only 6 deuterium atoms and, therefore, have lost 2 labels during the transformation. LC-MS analyses showed that the molecular weight of **3** and **4** is the same as the parent di-endoperoxide **2** (MW = 400; Fig. 2), and UV analyses indicate the presence of a substituted keto-ene moiety. The di-endoperoxide **2** as well as the novel metabolites **3** and **4** were absent in cells treated with the non-selective COX inhibitor indomethacin, and they were also absent in unstimulated cells (Fig. 2, bottom panel).

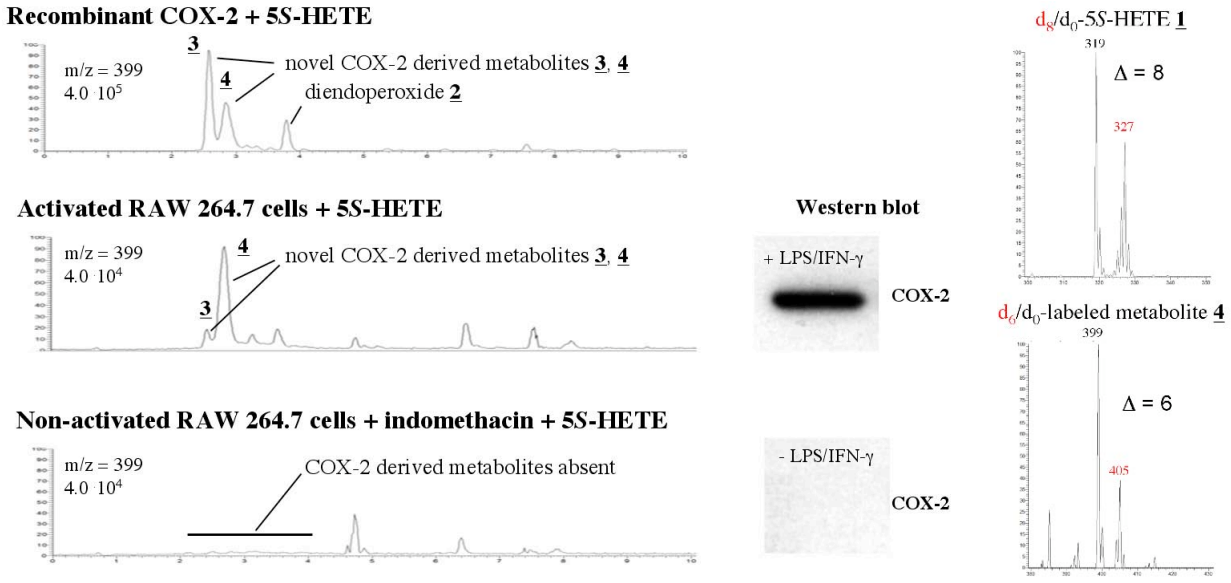


Fig. 2. LC-MS analyses of the transformation of 5-HETE by recombinant COX-2 (top panel), activated RAW264.7 cells (middle), and unactivated RAW264.7 cells in the presence of indomethacin (bottom). The ion traces for m/z 399 (negative ion mode) are shown at the same scale. On the right: LC-MS spectra of an approximate 1:1 mixture of d_0 and d_8 5-HETE incubated with activated RAW264.7 cells show that metabolites **4** and **3** (not shown) have lost 2 deuterium labels during the transformation. (The additional signals at m/z 326 and 325 in the d_0/d_8 5-HETE are derived from the substrate that was contaminated significantly with the d_7 and d_6 isomers.)

The results clearly indicate that the novel eicosanoids **3** and **4** are derived from conversion of 5-HETE **1** by COX-2, and that they are cellular transformation products of the di-endoperoxide **2** that we have previously characterized in vitro [1]. We have isolated sufficient amount of product **3** for characterization by ^{1}H and 2D NMR (Fig. 3).

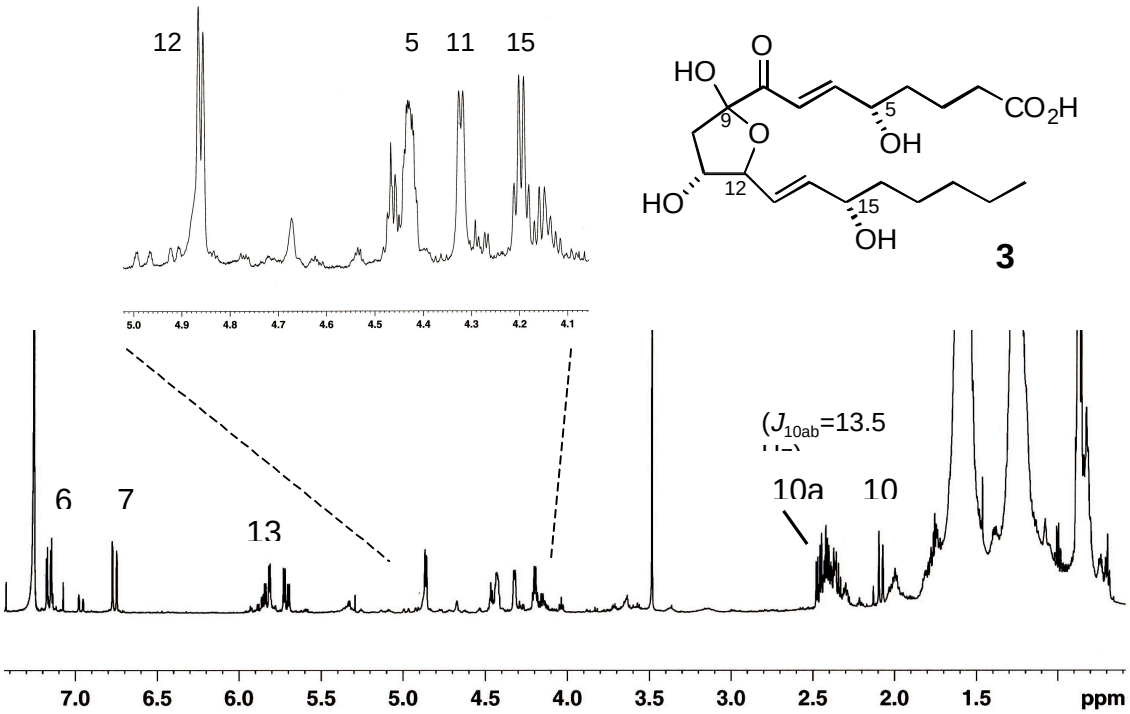


Fig. 3. ^{1}H NMR spectrum and chemical structure of **3** from the reaction of 5*S*-HETE with COX-2.

The ^{1}H and 2D NMR spectra (H,H-COSY, HSQC, and HMBC) outlined a structure of **3** as shown in Fig. 3. A prominent feature of the novel eicosanoid is the hydroxylated tetrahydrofuran ring of carbons 9 through 12. As discussed below, the hemiketal at carbon 9 gave an important clue about the mechanism of formation of this product.

The fact that **4** was more prominent than **3** in RAW264.7 cells treated with 5*S*-HETE (whereas rearrangement of the di-endoperoxide *in vitro* favors formation of **3**) pointed toward an enzymatic contribution in the formation of **4** from the di-endoperoxide. We reasoned that a prostaglandin synthase was involved in the formation, and decided to test the two prostaglandin D synthases (PGDS) first, i.e., the hematopoietic-type and the lipocalin-type PGDS. We cloned and expressed the hematopoietic PGDS (hPGDS) in *E. coli* and we purchased the lipocalin-type

PGDS (lPGDS) from Cayman Chemical. Fig. 4 shows LC-MS chromatograms of the analysis of the reaction of the di-endoperoxide with hPGDS and lPGDS.

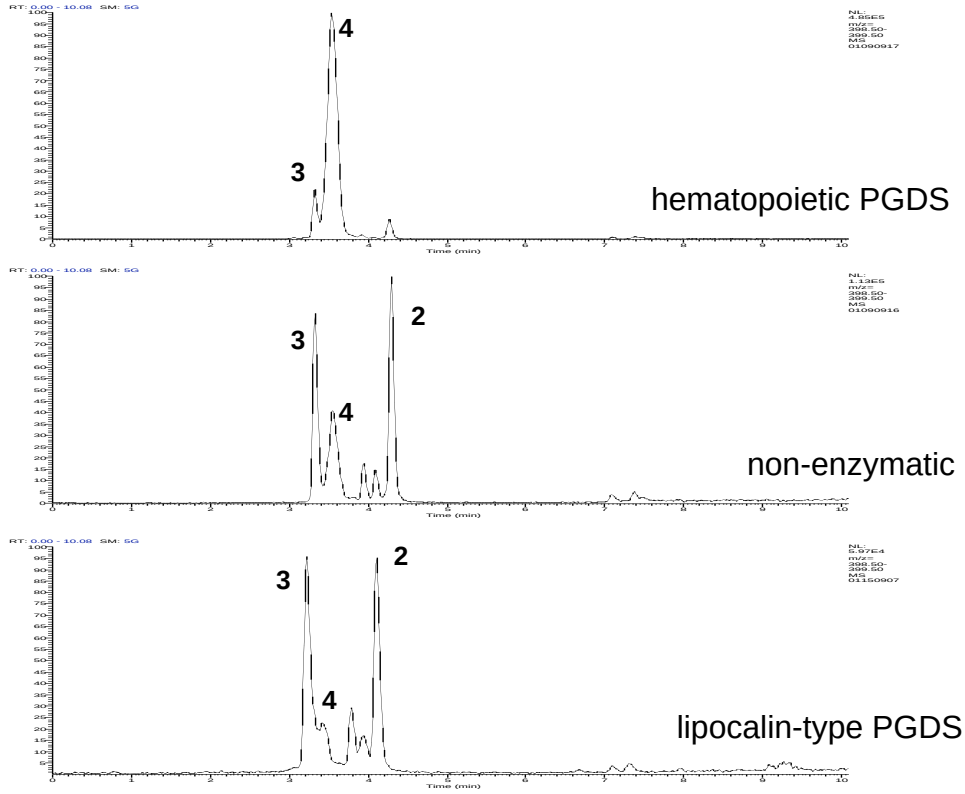


Fig. 4. LC-ESI-MS analysis of the transformation of the di-endoperoxide **2** by hematopoietic PGDS (top), lipocalin-type PGDS (bottom), and non-anzymatic (middle).

LC-MS analyses showed that the di-endoperoxide **2** is an efficient substrate for hPGDS forming **4** whereas the lipocalin-type PGDS does not react with **2**. We therefore used the consecutive transformation of 5*S*-HETE by COX-2 and hPGDS for large-scale preparation of **4** for analysis by NMR. The NMR data showed that **4** was an isomer of **3** showing a similarly hydroxylated tetrahydrofuran ring with a hemiketal carbon (Fig. 5). We reasoned that both **3** and **4** are derived from opening of the two endoperoxide rings of **2** as shown in Fig. 5.

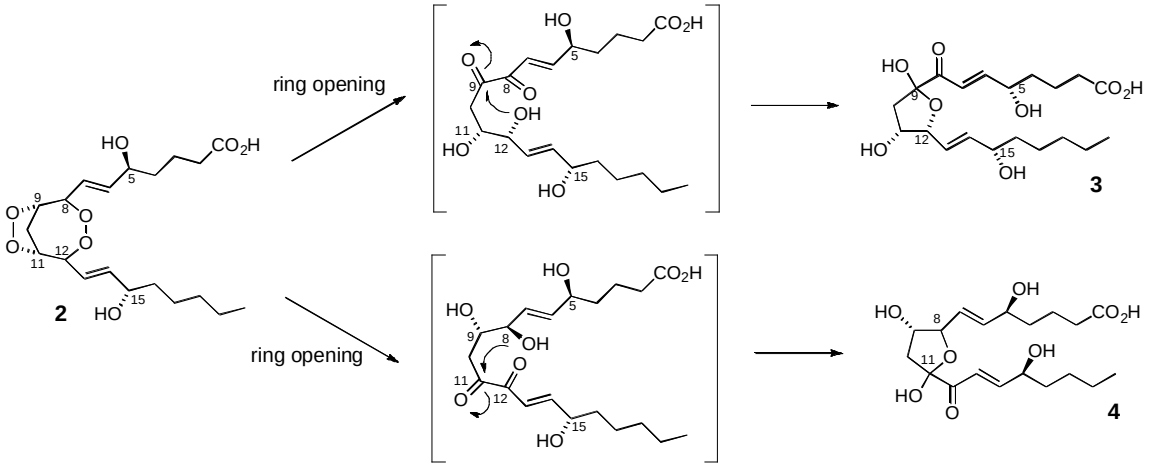


Fig. 5. Transformation of the di-endoperoxide **2** to the hemiketals **3** and **4**. The diketo-dihydroxy intermediates shown in the brackets have not been isolated. The transformations shown occur non-enzymatically, and in addition, the transformation from **2** to **4** in the lower panel is also catalyzed by the hematopoietic PGDS.

Task 3. To treat breast cancer cell lines with the novel metabolite and characterize its effect on differentiation, proliferation, and angiogenesis

This task was not completed due to the difficulties in identification of **3** and **4** and lack of availability of sufficient amounts for biological testing of their activities.

Key Research Accomplishments

- A new antibody for detection of human 5-LOX was generated and tested successfully.
- Two novel eicosanoids derived from the consecutive activities of 5-LOX and COX-2 were detected.
- The novel eicosanoids have a tetrahydrofuran ring in conjunction with a hemiketal moiety.
- Formation of one of the hemiketals from the di-endoperoxide is catalyzed by hematopoietic prostaglandin D synthase.

Reportable Outcomes

Mulugeta, S., Suzuki, T., Tejera Hernandez, N., Griesser, M., Boeglin, W.E., and Schneider, C. (2009) Identification and absolute configuration of dihydroxy-arachidonic acids formed by oxygenation of 5*S*-hydroxyeicosatetraenoic acid by native and aspirin-acetylated cyclooxygenase-2, submitted to *J. Lipid Res.*

Griesser, M., Boeglin, W.E., Suzuki, T., and Schneider, C. (2009) Convergence of the 5-LOX and COX-2 pathways. Heme-catalyzed cleavage of the 5*S*-HETE-derived di-endoperoxide into aldehyde fragments, *J. Lipid Res.*, in press.

An abstract reporting the results of task 2 was presented at the AACR Special Conference on Chemical and Biological Aspects of Inflammation and Cancer (October 14-17, 2008) organized by the American Association for Cancer Research:

Markus Griesser, Takashi Suzuki, and Claus Schneider: “Characterization of novel 5-lipoxygenase/cyclooxygenase-2-derived eicosanoids”. A copy of the abstract is attached as an appendix.

Conclusion

Our hypothesis of the cross-over of the 5-lipoxygenase and COX-2 pathways in breast cancer was supported by the detection of two novel eicosanoids derived from the consecutive

enzymatic conversion of arachidonic acid by both enzymes. The novel eicosanoids have a tetrahydrofuran ring in conjunction with a hemiketal moiety. Formation of one of the hemiketals from the di-endoperoxide is catalyzed by the enzyme hematopoietic prostaglandin D synthase

References

[1] Schneider, C., Boeglin, W. E., Yin, H., Stec, D. F., Voehler, M., Convergent oxygenation of arachidonic acid by 5-lipoxygenase and cyclooxygenase-2. *J. Am. Chem. Soc.* 2006, *128*, 720-721.

Appendices

Copies of the papers submitted to and in press at the *Journal of Lipid Research* as well as a copy of the abstract presented at the AACR meeting are attached.

Characterization of novel 5-lipoxygenase/cyclooxygenase-2-derived eicosanoids

Markus Griesser, Takashi Suzuki, and Claus Schneider

Division of Clinical Pharmacology, Department of Pharmacology, Vanderbilt University Medical Center, Nashville, TN 37232, USA

5-Lipoxygenase (5-LOX) and cyclooxygenase-2 (COX-2) initiate two separate biosynthetic pathways of lipid hormones (leukotrienes and prostaglandins, respectively) with distinct physiological roles in tissue homeostasis but also in inflammation and cancer. Both enzymes use arachidonic acid as the common substrate, and therefore the formation of the initial peroxide intermediate by 5-LOX or COX-2 is recognized as the committed step towards leukotriene or prostaglandin biosynthesis. We recently provided evidence for an unexpected biochemical interaction of the 5-LOX and COX-2 pathways: the 5-LOX product, 5*S*-hydroxyeicosatetraenoic acid (5*S*-HETE) is a selective and efficient substrate for the oxygenation by COX-2; the COX-1 isozyme does not react. *In vitro*, the major oxygenation product of 5*S*-HETE by COX-2 is a novel bicyclic di-endoperoxide. In activated RAW 264.7 cells a series of COX-2 derived metabolites of 5*S*-HETE was detected using LC-MS analyses. Isotopic labeling studies indicate that these eicosanoids were derived from the transformation of the intermediate di-endoperoxide. Further characterization of the cross-over of the 5-LOX and COX-2 pathways and its products could shed new light on the etiology and regulation of inflammatory diseases, and may also pave new ways for understanding the pathophysiology of other common diseases implicating 5-LOX and COX-2, like asthma and cancer.

Convergence of the 5-LOX and COX-2 pathways. Heme-catalyzed cleavage of the 5*S*-HETE-derived di-endoperoxide into aldehyde fragments

Markus Griesser, William E. Boeglin, Takashi Suzuki, Claus Schneider

Division of Clinical Pharmacology, Department of Pharmacology, Vanderbilt University Medical School, Nashville, Tennessee 37232, U.S.A.

correspondence address:
Claus Schneider, PhD, Division of Clinical Pharmacology, Department of Pharmacology, Vanderbilt University Medical School, RRB 572, 23rd Ave. S. at Pierce, Nashville, TN 37232-6602, U.S.A., Tel.: 615-343-9539, Fax: 615-322-4707, email: claus.schneider@vanderbilt.edu

Running footline: Transformation of arachidonic acid di-endoperoxide

Abbreviations:

APCI	atmospheric pressure chemical ionization
COX-2	cyclooxygenase-2
2,4-DNPH	2,4-dinitrophenylhydrazine
5*S*-HETE	5*S*-hydroxy-eicosatetra-6*E*,8*Z*,11*Z*,14*Z*-enoic acid
HHT	12*S*-hydroxy-heptadecatri-5*Z*,8*E*,10*E*-enoic acid
4-HNE	4-hydroxy-2*E*-nonenal
5-LOX	5-lipoxygenase
MDA	malondialdehyde
PG	prostaglandin

Abstract

Oxygenation of the 5-lipoxygenase product 5*S*-hydroxyeicosatetraenoic acid by cyclooxygenase-2 yields a bicyclic di-endoperoxide. The di-endoperoxide contains two peroxides spanning from carbons 9 to 11 and 8 to 12, and two hydroxyls at carbons 5 and 15 of arachidonic acid (Schneider C. et al., *J. Am. Chem. Soc.* 128 (2006), 720). Here, we report that treatment of the di-endoperoxide with hematin or ferrous chloride results in cleavage of both peroxide O-O bonds and of the bonds between the carbons that carry the peroxide groups, producing the aldehydes 4-hydroxy-2*E*-nonenal (4-HNE), 8-oxo-5*S*-hydroxy-6*E*-octenoic acid, and malondialdehyde (MDA). The hematin- and ferrous iron-catalyzed transformation of the di-endoperoxide proceeded with a similar yield of products as the cleavage of the prostaglandin endoperoxide PGH_2 to 12*S*-hydroxy-5*Z*,8*E*,10*E*-heptadecatrienoic acid and malondialdehyde. Chiral phase HPLC analysis of the 4-HNE cleavage product showed greater than 98% 4*S*, and thus established the *S* configuration of the 15-carbon of the di-endoperoxide that had not previously been assigned. This transformation of the 5-LOX/COX-2 derived di-endoperoxide invokes the possibility of a novel pathway to formation of the classic lipid peroxidation products 4-HNE and MDA.

Supplementary key words: 4-hydroxy-nonenal, malondialdehyde, lipid peroxide, aldehyde, prostaglandin

Introduction

The 5-lipoxygenase (5-LOX) product 5*S*-HETE is an efficient substrate for oxygenation by the so-called "inducible" form of prostaglandin H synthase, cyclooxygenase-2 (COX-2) (1). 5*R*-HETE is far less efficient as a substrate for COX-2, and COX-1,the isozyme that is regarded as the "housekeeping" form, does not react with either enantiomer of 5-HETE. During the transformation of 5*S*-HETE, COX-2 consumes 3 moles of oxygen and forms a di-endoperoxide that is structurally reminiscent of the prostaglandin endoperoxide (PGH_2) derived from double oxygenation of arachidonic acid by either COX isozyme (Fig. 1). The additional oxygen is incorporated as a peroxide that connects carbons 8 and 12, in place of the carbon-carbon bond that is part of the typical five-membered carbon ring of the prostaglandins (1).

Identification of an unstable peroxide product similar to PGH_2, but formed by consecutive action of 5-LOX and COX-2, has invoked the possibility of a cross-over of the leukotriene and prostaglandin biosynthetic pathways. The di-endoperoxide has been identified using *in vitro* biochemical transformation (1). Cross-over of the 5-LOX and COX-2 pathways has yet to be established to occur *in vivo*. The instability of the di-endoperoxide, however, impedes attempts aimed at direct detection of this product in cultured cells or animal tissue. The studies reported here were designed to predict and identify potential metabolites of the di-endoperoxide, with the future goal of using this information for the detection of metabolites that are indicative of formation of the di-endoperoxide as consecutive oxygenation products of arachidonic acid by 5-LOX and COX-2.

The non-enzymatic transformations of the arachidonic acid-derived endoperoxide PGH_2 include cleavage to malondialdehyde (MDA) and 12*S*-hydroxy-heptadecatri-5*Z*,8*E*,10*E*-enoic acid (HHT) as a minor reaction, and rearrangement to the prostaglandins PGE_2 and PGD_2 and the so-called levuglandins as major reaction (2, 3). Cleavage of the carbon chain is accelerated by the presence of ferrous iron (4, 5), heme (6), and it is also catalyzed by the enzyme thromboxane synthase (4, 7). Here, we describe the non-

enzymatic transformation of the 5*S*-HETE derived di-endoperoxide in the presence of heme and ferrous iron (Fe^{2+}) in aqueous solution. In addition, chiral analysis of one of the transformation products, 4-HNE, was used to determine the absolute configuration of the 15-carbon of the di-endoperoxide that had not been established as part of the original structural analysis (1).

Experimental Procedures

Synthesis of 5S-HETE

Racemic 5-HETE was synthesized following the method of Corey and Hashimoto (8). One g of arachidonic acid was dissolved in 12.85 ml of tetrahydrofuran and 6.4 ml of aqueous potassium bicarbonate (1.24 g) and cooled to 0°C. Potassium iodide (1.64 g) and iodine (4.74 g) were added sequentially to the solution, the flask was wrapped with aluminum foil and stirred in the dark at 4°C for 15 h. The solution was poured into ice-cold sodium thiosulfate solution (120 g in 140 ml of water), and extracted three times with 20 ml of pentane/diethyl ether (3/2). The organic phase was washed with 3.5% Na_2CO_3 solution and with saturated NaCl. The organic phase was filtered over a 50 g silica bond cartridge and eluted with hexane/diethyl ether (3/1).

The collected iodo lactone was evaporated to dryness and dissolved in 10 ml of dry benzene. A solution of 0.2 g of 1,5-diazabicyclo[5.4.0]undec-5-ene in 2 ml of benzene was added, and the solution was stirred at room temperature under argon overnight. After 17 h 1.14 g of powdered $CuSO_4$ x 5 H_2O was added and the solution was stirred for an additional 30 min. The mixture was diluted with 12 ml of hexane and filtered rapidly through a 5 g silica bond cartridge eluted 4-times with 20 ml of diethyl ether/hexane (3/1). The δ-lactone of 5-HETE was evaporated under a stream of nitrogen, and 3.5 ml of methanol and 0.43 g of triethylamine were added. The mixture was placed under argon and stirred at room temperature for 24 h. The solvent was evaporated and 5-HETE methyl ester (about 25% yield from arachidonic acid) was isolated using a 2 g silica bond cartridge eluted with hexane/diethyl ether (3/1). 5-HETE methyl ester was further purified by SP-HPLC using an Alltech Econosil Silica column (10 x 250 mm) eluted with a solvent of hexane/isopropanol (100/5, by vol.) at a flow rate of 4 ml/min and UV detection at 250 nm.

5*S*-HETE methyl ester was resolved from 5*R*-HETE methyl ester by chiral phase HPLC using a Chiralpak AD column (10 x 250 mm) eluted with a solvent of hexane/methanol (100/2, by vol.) at a flow rate of 3 ml/min and UV detection at 250 nm (9). The *R*

enantiomer of 5-HETE methyl ester elutes before the *S* enantiomer. For hydrolysis to the free acid a 4 mg aliquot of 5*S*-HETE methyl ester was dissolved in 2 ml of methanol and 100 µl of dichloromethane. One ml of 1 N KOH was added, and the solution was allowed to stand for 45 min. The organic solvents were evaporated under a stream of nitrogen, and the solution was acidified with 1 N HCl to pH 3. 5*S*-HETE was extracted into dichloromethane, washed three times with water, and evaporated to dryness. A solution of 5 mg/ml of 5*S*-HETE in methanol was prepared and stored at -80°C until further use.

Synthesis of the di-endoperoxide

In a 7 ml glass vial, 80 µl of a 20 µM solution of purified recombinant human COX-2 (10) were added to 2 ml of 100 mM Tris-HCl pH 8.0 containing 500 µM phenol and 1 µM hematin. After 2 min of incubation in the absence of substrate to allow for reconstitution of the holoenzyme, 50 µg of unlabeled 5*S*-HETE and 150,000 cpm of [1-^{14}C]-5*S*-HETE were added. The reaction was allowed to proceed for 5 min and was terminated by the addition of 16 µl of glacial acetic acid dissolved in 50 µl of methanol. The entire reaction mixture was applied to a 1-cc (30 mg) Waters Oasis HLB cartridge, washed with water, and eluted with methanol. The di-endoperoxide product was isolated by RP-HPLC using a Waters Symmetry C18 column (4.6 x 250 mm) eluted with a gradient of 20% acetonitrile to 70% acetonitrile in 0.01% aqueous acetic acid in 20 min at a flow rate of 1 ml/min. Elution of the products was monitored at UV 206 nm. The isolated yield of the di-endoperoxide was calculated to be about 15%.

Transformation of the di-endoperoxide with hematin

For transformation of the di-endoperoxide with heme 1 ml of 100 mM Tris-HCl buffer pH 8.0 containing 500 µM phenol and 1 µM hematin was used. 40 µl of COX-2 solution was added (0.6 µM final concentration), followed by the addition of 20 µg of 5*S*-HETE. After 5 min at room temperature, 1 ml of a 500 µM solution of hematin was added and the reaction was continued for 1 h. The reaction mixture was either acidified with 1 N HCl to pH 3 and extracted, or 1 ml of a solution of 2,4-DNPH (0.3 mg/ml 0.2 N HCl) was added and the reaction was incubated in the dark at room temperature for an additional 1 h, followed by acidification to pH 3. The reaction mixtures were loaded on a

1-cc (30 mg) Waters Oasis HLB cartridge, washed extensively with water, and eluted with acetonitrile. For heme-catalyzed transformation of PGH_2 the same protocol was followed except for using 20 µg of arachidonic acid instead of 5*S*-HETE. The transformation of 20 µg of 5*S*-HETE or arachidonic acid with 1 ml of 500 µM hematin in the absence of COX-2 was used as control reaction.

Transformation of the di-endoperoxide with $FeCl_2$

About 5 µg of the isolated [1-^{14}C]-labeled di-endoperoxide (≈15,000 cpm) were mixed immediately after collection off RP-HPLC with 1 ml of a freshly prepared solution of $FeCl_2$ in water (20 mM). After 1 min, the products were loaded onto a Waters Oasis HLB cartridge, washed with water, and eluted with methanol.

Autoxidation of 5S-HETE

One mg of 5*S*-HETE was evaporated to dryness in a plastic tube and incubated at 37°C for one hour. The entire autoxidation mixture was then injected using a Waters Symmetry C18 column (4.6 x 250 mm) eluted with a gradient of 20% acetonitrile to 70% acetonitrile in 0.01% aqueous acetic acid in 20 min followed by 10 min of isocratic elution at a flow rate of 1 ml/min. The peak eluting at 4.1 min (corresponding to 8-oxo-5-hydroxy-6*E*-octenoic acid) was collected, extracted into dichloromethane, and dissolved in $CDCl_3$ for NMR analysis. The ^{1}H and H,H-COSY NMR spectra were recorded using a Bruker AV-II-600 MHz spectrometer. Chemical shifts are calibrated to δ = 7.25 ppm for the remaining $CHCl_3$ in the solvent. The isolated compound was further transformed to the methyl ester derivative with ethereal diazomethane, followed by treatment with methyloxime hydrochloride in pyridine to give the methoxime derivative, and finally with N,O-*bis*(trimethylsilyl) trifluoroacetamide. The methyl ester, methoxime, TMS ether derivative was analyzed by GC-MS in the electron impact mode (70 eV) using a Thermo Finnigan Trace DSQ instrument equipped with a 30 m HP5 column (0.25 mm i.d., film thickness 0.25 µm). The temperature was programmed from 125°C (hold for 1 min) to 300°C at 20°C/min.

Synthesis of 1-pyrazole

One mg of 1,1,3,3-tetraethoxypropane was dissolved in 1 ml of 0.1 N HCl and incubated at 50°C for 1 h. The concentration of MDA in the solution was calculated assuming 100% conversion. To 100 µl of the solution, 1 ml of DNPH reagent (0.3 mg/ml 0.2 N HCl) was added and incubated in the dark at room temperature for 1 h. The solution was extracted using a Waters Oasis HLB cartridge as described above. A single product peak corresponding to DNPH-derivatized MDA (1-pyrazole) was detected using HPLC-DAD analysis.

HPLC analyses

Transformation of the di-endoperoxide and PGH_2 was analyzed using a Waters Symmetry C18 column (4.6 x 250 mm; 5 µm) eluted with a gradient of 20% acetonitrile to 70% acetonitrile in 0.01% aqueous acetic acid in 20 min followed by 10 min of isocratic elution at a flow rate of 1 ml/min. Elution of the products was monitored using an Agilent 1200 diode array detector. For analysis of the transformation products of [1-^{14}C]-labeled di-endoperoxide, the effluent of the diode array detector was coupled on-line to a Radiomatic A100 radioactive flow detector. For detection of 1-pyrazole the same HPLC-diode array system was used except the gradient was run from 20% acetonitrile to 80% acetonitrile in 0.01% aqueous acetic acid in 20 min followed by 5 min of isocratic elution and 5 min of washing with 100% methanol at a flow rate of 1 ml/min. LC-MS analysis of the 1-pyrazole was performed using a Thermo Finnigan LTQ ion trap mass spectrometer. The sample was resolved using a Waters Symmetry Shield RP-18 column (2.1 x 100 mm; 3 µm), eluted at 0.3 ml/min flow rate with a linear gradient of 5% acetonitrile to 95% acetonitrile in water (containing 10 mM NH_4OAc each) within 10 min. The APCI interface was operated at a vaporizer temperature of 300°C and a tube lens voltage of -80 V. Negative ions were monitored over a range of 150-1200 mass units.

Chiral analysis of 4-HNE

The absolute configuration of 4-HNE was analyzed following a previously described method (11). 4-HNE derived from the $FeCl_2$-catalyzed transformation of the di-endoperoxide was collected using SP-HPLC and evaporated to dryness under a stream of

nitrogen. The sample was dissolved in 10 µl of acetonitrile and treated with 20 µl of a solution of methoxyamine hydrochloride (10 mg/ml) in pyridine at room temperature overnight. In parallel, 10 µg of racemic 4-HNE were treated with methoxyamine hydrochloride in a similar way. The reaction mixtures were diluted with 500 µl of dichloromethane, and washed three times with an equivalent volume of water. The organic solvent was evaporated, and the *syn* and *anti* isomers were separated by RP-HPLC using a Waters Symmetry C18 column (4.6 x 250 mm) eluted with a solvent of acetonitrile/water/acetic acid (50/50/0.01, by vol.) at a flow rate of 1 ml/min and UV detection at 235 nm. The later eluting isomer was analyzed by chiral phase HPLC using a Chiralpak AD column (4.6 x 250 mm) eluted with a solvent of hexane/ethanol (90/10, by vol.) at a flow rate of 1 ml/min and UV detection at 235 nm.

Results

Oxygenation of 5*S*-HETE by COX-2 resulted in formation of the di-endoperoxide which was detected using RP-HPLC analysis as described previously (Fig. 2A) (1). The di-endoperoxide (eluting at 14.6 min retention time) contains two isolated double bonds resulting in a UV chromophore with maximum absorbance around 200 nm and noticeable extension of the absorbance beyond 240 nm. A by-product of the reaction was identified as 5,15-diHETE by comparison of the UV spectrum and chromatographic retention time with an authentic standard. For transformation of the di-endoperoxide the crude product mixture was treated with 500 µl of a 500 µM hematin solution. After 1 h of incubation, the products were extracted and re-analyzed by RP-HPLC. The di-endoperoxide was transformed quantitatively, and two prominent peaks were formed with elution times of 4.1 min and 14.7 (Fig. 2B). The UV spectra of both products were superimposable and indicative of a conjugated oxo-ene chromophore showing a maximum absorbance at 223 nm in RP-HPLC column solvent. When the HPLC-isolated [1-^{14}C]-labeled di-endoperoxide was treated with $FeCl_2$ instead of hematin the same peaks were detected using diode array detection (Fig. 2C). Analysis of the products using an on-line coupled radioactive flow detector showed that only the first peak at 4.1 min contained radioactivity whereas the second peak at 14.7 min was not radiolabeled (Fig. 2D). No significant other labeled or unlabeled products were detected.

The earlier eluting product (4.1 min retention time) was identified as 8-oxo-5*S*-hydroxy-6*E*-octenoic acid based on comparison to a reference compounds that was prepared as follows. 8-Oxo-5*S*-hydroxy-6*E*-octenoic acid was predicted to be formed upon cleavage of the 8,9-double bond during autoxidative transformation of 5*S*-HETE, analogous to the cleavage of 9*S*-hydroxy-10*E*,12*Z*-octadecadienoic acid to 12-oxo-9*S*-hydroxy-10*E*-dodecenoic acid (11). Autoxidation of 5*S*-HETE as a thin film gave the predicted peak at 4.1 min retention time. Using this route of synthesis, the product was formed with higher yield than via heme-catalyzed breakdown of the di-endoperoxide. Therefore, autoxidation of a 1 mg-aliquot of 5*S*-HETE was used to generate sufficient material for spectroscopic characterization. ^{1}H NMR analysis showed a doublet signal at 9.58 ppm for the aldehyde

proton (H8) that was coupled to H7 with a coupling constant $J_{7,8}$ = 7.9 Hz (δ 6.32 ppm; ddd; $J_{7,5}$ = 1.5 Hz). H7 and H6 (δ 6.81 ppm; dd) form a *trans* double bond ($J_{7,6}$ = 15.7 Hz). In the H,H-COSY spectrum H6 showed a cross-peak with H5 (J = 4.6 Hz) which appeared as a multiplet signal at 4.47 ppm. For GC-MS analysis the methyl ester, methoxime, TMS ether derivative was prepared. The electron impact analysis (70 eV) of the *syn* and *anti* isomers gave essentially the same mass spectra with major fragment ions at *m/z* 256 ($[M-31]^+$, loss of OMe from the methyl ester or methoxime, respectively; 98% intensity) and *m/z* 186 ($[CH\text{-}OTMS\text{-}CH\text{-}CH\text{-}CH{=}NOCH_3]^+$; 95% intensity), respectively. The $[M]^+$ peak at *m/z* 287 showed 5% intensity, and the base peak was found at *m/z* 73, indicative of the TMS-derivative. The *S*-configuration of the 5-carbon is assumed to be unchanged from the 5*S*-HETE starting material (11). The later peak eluting at 14.7 min was identified as 4-HNE based on its identical UV spectrum and co-elution with an authentic standard using both RP-HPLC and SP-HPLC (11).

Semi-quantitative analysis of the formation of the two aldehyde products after 1 h of treatment with hematin gave about 10% molar yield of 4-HNE and 3-5% molar yield of the 8-oxo acid. Shorter reaction times resulted in incomplete transformation of the di-endoperoxide. Reaction times longer than 1 h resulted in a decline of the yield of the aldehydes, presumably due to an increased prevalence of secondary transformation reactions. In the absence of hematin or $FeCl_2$ cleavage of the carbon chain was markedly reduced, and the di-endoperoxide was predominantly transformed to two different products instead (M.G. and C.S., unpublished observation).

The possibility that the aldehydes were derived from 5*S*-HETE directly or a reaction by-product rather than from the di-endoperoxide was excluded based on three observations. First, in all reactions sufficient amount of COX-2 was used to achieve near complete conversion of 5*S*-HETE or arachidonic acid, respectively, prior to the addition of hematin or $FeCl_2$ (cf. Fig 2A). Secondly, when the HPLC-isolated di-endoperoxide was treated with $FeCl_2$ the two aldehydes were detected as the almost exclusive products (cf. Fig 2C). Thirdly, when 20 μg of 5*S*-HETE were incubated in the absence of COX-2 with the hematin solution for 1 h neither the aldehyde products nor any other abundant UV-active

polar products were detected although only about 10% of the intact 5*S*-HETE was recovered (not shown). The absence of UV-detectable polar products indicated that 5*S*-HETE was transformed to products that lack UV absorbance or to products of lesser polarity than the starting material, possibly including dimeric or oligomeric products.

Cleavage of the 20-carbon di-endoperoxide to give the 9- and 8-carbon aldehydes, 4-HNE and 8-oxo-5-hydroxy-6*E*-octenoic acid, implicated that malondialdehyde (MDA) was formed as the third product of heme-catalyzed transformation. MDA was detected as its 1-pyrazole derivative using the 2,4-DNPH reagent to trap the aldehyde (12). Incubation of 20 μg of 5*S*-HETE with COX-2 and the subsequent transformation with hematin was performed as described above and followed by immediate treatment of the crude reaction mixture with 2,4-DNPH. MDA was detected as the 1-pyrazole derivative of 2,4-DNPH using LC-MS with APCI ionization (Figs. 3A and 3B). The molar yield of MDA was between 5-10% as determined using RP-HPLC analyses (Fig. 3C). A similar amount of MDA was detected when 20 μg of arachidonic acid were oxygenated to PGH_2 followed by treatment with heme and DNPH reagent (Fig. 3D). Thus, under the conditions used the di-endoperoxide and PGH_2 were about equally effective in formation of MDA when treated with hematin. Formation of MDA in either case was dependent on an endoperoxide intermediate because incubation of arachidonic acid with hematin and DNPH reagent without prior reaction with COX-2 failed to show formation of MDA (Fig. 3E). This excluded the possibility that MDA was derived from another, unrelated transformation of arachidonic acid.

The 4-HNE cleavage product is derived from the methyl end of the di-endoperoxide, and therefore, the absolute configuration of the 4-hydroxyl group is equivalent to the configuration of the 15-carbon of the di-endoperoxide. The configuration of this chiral center of the di-endoperoxide had not been established previously (1). 4-HNE formed by hematin-catalyzed transformation of the di-endoperoxide was isolated and reacted to its methoxime derivatives (*syn* and *anti* isomers) to enable analysis of the absolute configuration using chiral phase HPLC. The later eluting double bond isomer was isolated using RP-HPLC and then resolved into its enantiomers using a Chiralpak AD

column. 4-HNE was greater than 98% of the *S*-configuration providing evidence that the 15-carbon of the di-endoperoxide has the *S*-configuration (Fig. 4).

Discussion

Heme-catalyzed transformation of the 5-LOX/COX-2 derived di-endoperoxide of arachidonic acid resulted in chain cleavage between the carbons carrying the peroxide groups and also between the O-O bonds of both peroxide groups (Fig. 5A). The resulting aldehyde fragments were identified as 8-oxo-5-hydroxy-6*E*-octenoic acid, 4-HNE, and MDA. The equivalent transformation of the arachidonic acid-derived prostaglandin endoperoxide PGH_2 gives MDA and the 17-carbon fragment, HHT (Fig. 5B). Cleavage of the 8,9 and 11,12 carbon bonds and of the 9,11-peroxide occurs similarly in both the di-endoperoxide and PGH_2. But whereas in PGH_2 C-8 and C-12 are connected directly as part of the cyclopentane (prostane) ring, in the di-endoperoxide C-8 and C-12 are connected through the second peroxide group, and thus cleavage of the 8,12-peroxide leads to a further fragmentation of the carbon chain. The lack of a five-membered carbon-ring precludes the possibility that the di-endoperoxide is transformed into analogues of the levuglandins or isoketals that are formed by cleavage of either the 9,10 or 10,11 carbon bond of PGH_2 while the 8,12 carbon bond stays intact (3, 13).

The lipid aldehyde 4-HNE has gained significance and notoriety as one of the major cytotoxic products formed during lipid peroxidation (14), but it can also serve as a bona fide signaling molecule (15, 16). The chemical mechanisms that account for the formation of 4-HNE from polyunsaturated fatty acids during lipid peroxidation have been interrogated for some time, and there have been suggestions for several pathways (17). 4-HNE is derived from different fatty acid precursors, including linoleic and arachidonic acids, and it is likely a common end point of multiple chemical reactions rather than the product of one single pathway. A recent review of mechanisms that are involved in formation of 4-HNE suggested cleavage of a 1,2-diperoxy moiety as a possibility of carbon chain fission and aldehyde generation (18). The di-endoperoxide contains two such 1,2-peroxide moieties, and the aldehydes we have identified result from the predictable cleavage sites (Fig. 5). It is a remarkable structural coincidence that 4-HNE is formed as one of the cleavage products. The question whether the di-endoperoxide could be a precursor for formation of 4-HNE (and MDA) during lipid peroxidation *in vivo*

remains to be addressed. Although the di-endoperoxide has been identified originally as an enzymatic product (1), there is also the mechanistic possibility of formation during autoxidation of arachidonic acid or 5-HETE. Studies on the autoxidative transformation of arachidonic acid have shown that essentially all eicosanoids formed enzymatically can likewise be produced by non-enzymatic pathways (e.g., (19, 20)), and the same might hold true for the di-endoperoxide.

The other large cleavage product, 8-oxo-5-hydroxy-6*E*-octenoic acid, is a γ-hydroxylated α,β-unsaturated aldehyde like 4-HNE (Fig. 5). The same compound, when esterified to the phospholipid backbone, has been identified as one of the targets recognized by the CD36 scavenger receptor (21, 22). As discussed above, if there were autoxidative formation of the di-endoperoxide it would likely occur in the membrane with the carboxyl group esterified to the phospholipid headgroup. Breakdown of the esterified di-endoperoxide would result in the phospholipid aldehyde as identified by Podrez and co-workers (21). Again, it will be interesting to see whether the di-endoperoxide could be an intermediate in the formation of this biologically active aldehyde.

One of the goals of this study was to generate and identify products that could serve as indicators of the cross-over of the 5-LOX and COX-2 pathways *in vivo* by using the model of heme-catalyzed transformation of the di-endoperoxide. Since the three major products formed, 4-HNE, MDA, and 8-oxo-5-hydroxy-6*E*-octenoic acid, can be derived through other pathways of enzymatic and/or non-enzymatic lipid peroxidation as well, they do not serve for this purpose. Nevertheless, we attempted to compare the levels of 4-HNE in intact activated murine RAW264.7 cells treated with and without 5*S*-HETE but could not see a difference (data not shown). ESI-LC-MS analyses of 8-oxo-5-hydroxy-6*E*-octenoic acid in cells were hampered by the unexpectedly low sensitivity for detection of this compound.

We used the transformation to 4-HNE as a strategy to indirectly determine the absolute configurations of the 15-carbon of the di-endoperoxide. Although oxygenation at the 15-carbon in prostaglandin biosynthesis occurs with very high precision in the *S*-

configuration (23, 24), there are several well documented examples where COX-2 catalyzes a specific oxygenation in the 15*R*-configuration. For example, aspirin-treated COX-2 specifically forms 15*R*-HETE (25), and certain mutations in the active site lead to formation of 15*R*-configuration prostaglandins (26) and other 15*R*-oxygenated products (10). In the latter case, inversion of the oxygenation at C-15 was likely dependent on the conformation of the reacting intermediate in the active site (10). It was, therefore, an open question whether the third oxygenation would occur in the *R*- or *S*-configuration. Our results provide evidence that the configuration of C-15 of the di-endoperoxide is >98% *S*.

Acknowledgements

This work was supported by NIH grants GM076592 and GM15431, and the Department of Defense Breast Cancer Research Program (BC063074).

References

1. Schneider, C., W. E. Boeglin, H. Yin, D. F. Stec, and M. Voehler. 2006. Convergent oxygenation of arachidonic acid by 5-lipoxygenase and cyclooxygenase-2. *J. Am. Chem. Soc.* **128**: 720-721.

2. Hamberg, M., and B. Samuelsson. 1967. Oxygenation of unsaturated fatty acids by the vesicular gland of sheep. *J. Biol. Chem.* **242**: 5344-5354.

3. Salomon, R. G., and D. B. Miller. 1985. Levuglandins: isolation, characterization, and total synthesis of new secoprostanoid products from prostaglandin endoperoxides. *Adv. Prostaglandin Thromboxane Leukot. Res.* **15**: 323-326.

4. Hamberg, M., and B. Samuelsson. 1974. Prostaglandin endoperoxides. Novel transformations of arachidonic acid in human platelets. *Proc. Natl. Acad. Sci. USA* **71**: 3400-3404.

5. Hammarberg, T., M. Hamberg, A. Wetterholm, H. Hansson, B. Samuelsson, and J. Z. Haeggstrom. 2009. Mutation of a critical arginine in microsomal Prostaglandin E Synthase-1 shifts the isomerase activity to a reductase activity that converts prostaglandin H2 into prostaglandin F2α. *J. Biol. Chem.* **284**: 301-305.

6. Nugteren, D. H., and E. Hazelhof. 1973. Isolation and properties of intermediates in prostaglandin biosynthesis. *Biochim. Biophys. Acta* **326**: 448-461.

7. Diczfalusy, U., P. Falardeau, and S. Hammarstrom. 1977. Conversion of prostaglandin endoperoxides to C17-hydroxy acids catalyzed by human platelet thromboxane synthase. *FEBS Lett.* **84**: 271-274.

8. Corey, E. J., and S. Hashimoto. 1981. A practical process for large-scale synthesis of (S)-5-hydroxy-6-trans-8,11,14,cis-eicosatetraenoic acid (5-HETE). *Tetrahed. Lett.* **22**: 299-302.

9. Schneider, C., W. E. Boeglin, and A. R. Brash. 2000. Enantiomeric separation of hydroxy-eicosanoids by chiral column chromatography: effect of the alcohol modifier. *Anal. Biochem.* **287**: 186-189.

10. Schneider, C., W. E. Boeglin, and A. R. Brash. 2004. Identification of two cyclooxygenase active site residues, leucine-384 and glycine-526, that control carbon ring cyclization in prostaglandin biosynthesis. *J. Biol. Chem.* **279**: 4404-4414.

11. Schneider, C., K. A. Tallman, N. A. Porter, and A. R. Brash. 2001. Two distinct pathways of formation of 4-hydroxynonenal. Mechanisms of nonenzymatic transformation of the 9- and 13-hydroperoxides of linoleic acid to 4-hydroxyalkenals. *J. Biol. Chem.* **276**: 20831-20838.

12. Bakalova, R., M. Mileva, C. Kotsev, V. Bardarov, and S. Ribarov. 2000. Determination of malondialdehyde in biological samples by solid-phase extraction and high-performance liquid chromatography. *Methods Find Exp. Clin. Pharmacol.* **22**: 267-269.

13. Brame, C. J., R. G. Salomon, J. D. Morrow, and L. J. Roberts, 2nd. 1999. Identification of extremely reactive gamma-ketoaldehydes (isolevuglandins) as products of the isoprostane pathway and characterization of their lysyl protein adducts. *J. Biol. Chem.* **274**: 13139-13146.

14. Benedetti, A., M. Comporti, and H. Esterbauer. 1980. Identification of 4-hydroxynonenal as a cytotoxic product originating from the peroxidation of liver microsomal lipids. *Biochim. Biophys. Acta* **620**: 281-296.

15. Nakashima, I., W. Liu, A. A. Akhand, K. Takeda, Y. Kawamoto, M. Kato, and H. Suzuki. 2003. 4-hydroxynonenal triggers multistep signal transduction cascades for suppression of cellular functions. *Mol. Aspects Med.* **24**: 231-238.

16. Dwivedi, S., A. Sharma, B. Patrick, R. Sharma, and Y. C. Awasthi. 2007. Role of 4-hydroxynonenal and its metabolites in signaling. *Redox Rep.* **12**: 4-10.

17. Porter, N. A., and W. A. Pryor. 1990. Suggested mechanisms for the production of 4-hydroxy-2-nonenal from the autoxidation of polyunsaturated fatty acids. *Free Radical Biol. Med.* **8**: 541-543.

18. Schneider, C., N. A. Porter, and A. R. Brash. 2008. Routes to 4-hydroxynonenal: fundamental issues in the mechanisms of lipid peroxidation. *J. Biol. Chem.* **283**: 15539-15543.

19. Gao, L., W. E. Zackert, J. J. Hasford, M. E. Danekis, G. L. Milne, C. Remmert, J. Reese, H. Yin, H. H. Tai, S. K. Dey, N. A. Porter, and J. D. Morrow. 2003. Formation of prostaglandins E2 and D2 via the isoprostane pathway: a mechanism for the generation of bioactive prostaglandins independent of cyclooxygenase. *J. Biol. Chem.* **278**: 28479-28489.

20. Roberts, L. J., 2nd, and J. P. Fessel. 2004. The biochemistry of the isoprostane, neuroprostane, and isofuran pathways of lipid peroxidation. *Chem. Phys. Lipids* **128**: 173-186.

21. Podrez, E. A., E. Poliakov, Z. Shen, R. Zhang, Y. Deng, M. Sun, P. J. Finton, L. Shan, M. Febbraio, D. P. Hajjar, R. L. Silverstein, H. F. Hoff, R. G. Salomon, and S. L. Hazen. 2002. A novel family of atherogenic oxidized phospholipids promotes macrophage foam cell formation via the scavenger receptor CD36 and is enriched in atherosclerotic lesions. *J. Biol. Chem.* **277**: 38517-38523.

22. Podrez, E. A., E. Poliakov, Z. Shen, R. Zhang, Y. Deng, M. Sun, P. J. Finton, L. Shan, B. Gugiu, P. L. Fox, H. F. Hoff, R. G. Salomon, and S. L. Hazen. 2002. Identification of a novel family of oxidized phospholipids that serve as ligands for the macrophage scavenger receptor CD36. *J. Biol. Chem.* **277**: 38503-38516.

23. Nugteren, D. H., D. A. Van Dorp, S. Bergstrom, M. Hamberg, and B. Samuelsson. 1966. Absolute configuration of the prostaglandins. *Nature* **212**: 38-39.
24. Schneider, C., D. A. Pratt, N. A. Porter, and A. R. Brash. 2007. Control of oxygenation in lipoxygenase and cyclooxygenase catalysis. *Chem. Biol.* **14**: 473-488.
25. Holtzman, M. J., J. Turk, and L. P. Shornick. 1992. Identification of a pharmacologically distinct prostaglandin H synthase in cultured epithelial cells. *J. Biol. Chem* **267**: 21438-21445.
26. Schneider, C., W. E. Boeglin, J. J. Prusakiewicz, S. W. Rowlinson, L. J. Marnett, N. Samel, and A. R. Brash. 2002. Control of prostaglandin stereochemistry at the 15-carbon by cyclooxygenases-1 and 2. A critical role for serine 530 and valine 349. *J. Biol. Chem.* **277**: 478-485.

Figure legends

Fig. 1. Comparison of the reaction of COX-2 with (A) 5*S*-HETE and (B) arachidonic acid forming a di-endoperoxide product or the prostaglandin endoperoxide (PGH_2), respectively. The relative configuration of the seven- or five-membered ring is the same in the di-endoperoxide and PGH_2, respectively. The absolute configuration of carbons 8 and 12 is 8*S* and 12*S* in the di-endoperoxide whereas it is 8*R* and 12*R* in PGH_2. Carbons 9 and 11 are 9*S* and 11*R* in both products. The configuration of C-15 of the di-endoperoxide had not been determined previously and was assigned in this study.

Fig. 2. RP-HPLC and UV analyses of the di-endoperoxide and the transformation reactions with heme and $FeCl_2$. (A) The di-endoperoxide (retention time 14.6 min) is the major product of the COX-2 catalyzed transformation of 5*S*-HETE. The UV spectrum (inset) shows the relative absorbance of the di-endoperoxide at 205 nm and 220 nm. The amount of COX-2 used was sufficient to achieve >95% conversion of the substrate. (B) 8-Oxo-5-hydroxy-6*E*-octenoic acid and 4-hydroxynonenal (4-HNE) were the major products detected upon heme-catalyzed transformation of the crude reaction shown in panel A. (C, D) The HPLC-purified [1-^{14}C]-labeled di-endoperoxide was treated with $FeCl_2$ for 1 min. The products were extracted and analyzed using RP-HPLC with a diode array detector (panel C) and a radioactive flow detector connected on-line (panel D). The inset in panel C shows the identical UV spectra of 8-oxo-5-hydroxy-6*E*-octenoic acid and 4-HNE with λ_{max} at 223 nm. Chromatograms recorded at 205 nm (solid line) and 220 nm (dashed line) are shown in panels A-C to illustrate the relative abundance of the products at the two wavelengths.

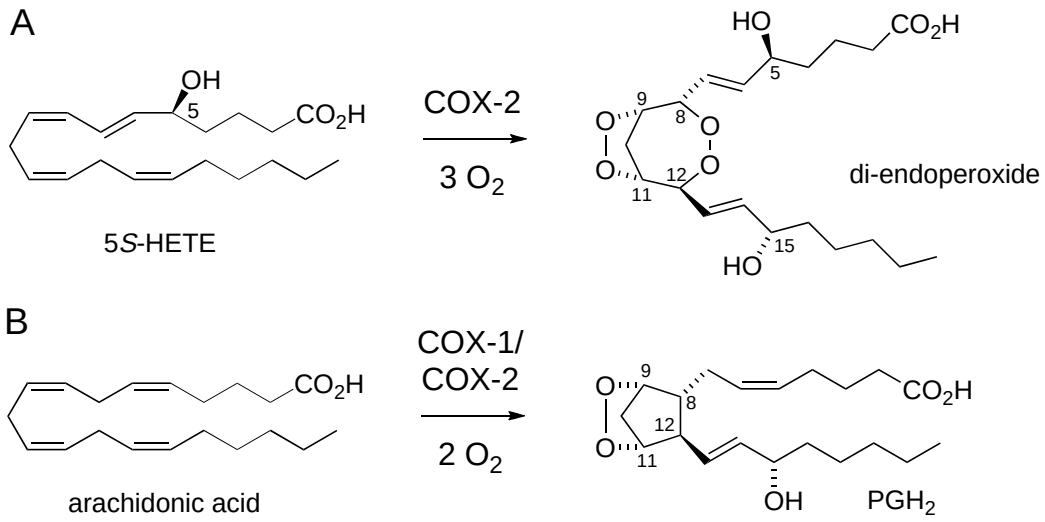
Fig. 3. Analysis of MDA formed by heme-catalyzed transformation of the di-endoperoxide and PGH_2. (A) LC-APCI analysis of a reaction of COX-2 with 20 μg of 5*S*-HETE followed by treatment with heme (1 h) and 2,4-DNPH reagent (1 h). The ion trace of *m*/*z* 234 for the 1-pyrazole derivative of 2,4-DNPH, recorded in negative ion

mode, is shown. (B) LC-APCI mass spectrum of the peak at 6.7 min retention time in panel A. The base peak shows the molecular ion $[M-H]^-$ of the 1-pyrazole of 2,4-DNPH. The signal at *m*/*z* 204 is due to loss of a fragment of 30 mass units, possibly $[N_2H_2]$ or [NO], form the parent ion. (C) The same reaction of 20 μg of 5*S*-HETE with COX-2 followed by treatment with heme and 2,4-DNPH reagent was analyzed using RP-HPLC with diode array detection. The back shoulder of the 1-pyrazole peak is due to an unidentified compound. (D) A similar amount of the 1-pyrazole derivative was detected when 20 μg of arachidonic acid were reacted with COX-2 followed by treatment with heme and DNPH reagent. (E) The 1-pyrazole derivative was not detected when arachidonic acid was treated with heme and DNPH reagent in the absence of COX-2. Equal aliquots of the transformation reactions were injected. The chromatograms shown in panels C-E were recorded at UV 205 nm. The chromatographic conditions used for panels A and C-E, respectively, are described in "Experimental Procedures".

Fig. 4. Chiral-phase HPLC analysis of 4-HNE derived from the di-endoperoxide. 4-HNE derived from the di-endoperoxide (A) and a racemic standard (B) were derivatized with methoxyamine hydrochloride, and analyzed by chiral phase HPLC using a Chiralpak AD column as described in Experimental Procedures. The elution order of the 4-HNE enantiomers on the chiral column had been established previously using CD spectroscopy of the methoxime derivatives (11).

Fig. 5. Transformation of the di-endoperoxide (A) and PGH_2 (B) by heme or $FeCl_2$.

Fig. 1


A
OH
5
CO2H
5S-HETE
COX-2
3 O2
HO
CO2H
5
9
8
O
O
O
O
12
11
15
HO
di-endoperoxide
B
CO2H
arachidonic acid
COX-1/
COX-2
2 O2
9
8
12
O
O
11
CO2H
OH
PGH2

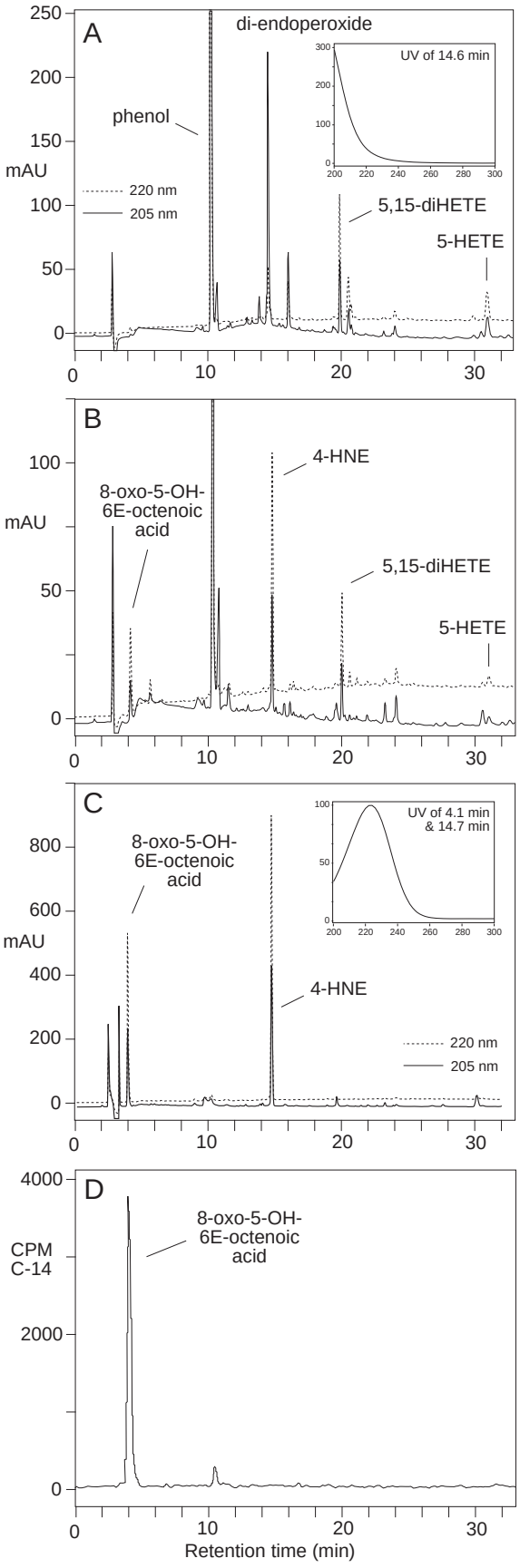

A
di-endoperoxide
UV of 14.6 min
phenol
220 nm
205 nm
5,15-diHETE
5-HETE
mAU
B
4-HNE
8-oxo-5-OH-
6E-octenoic
acid
5,15-diHETE
5-HETE
mAU
C
UV of 4.1 min
& 14.7 min
8-oxo-5-OH-
6E-octenoic
acid
4-HNE
220 nm
205 nm
mAU
D
8-oxo-5-OH-
6E-octenoic
acid
CPM
C-14
Retention time (min)

Fig. 2

Fig. 3

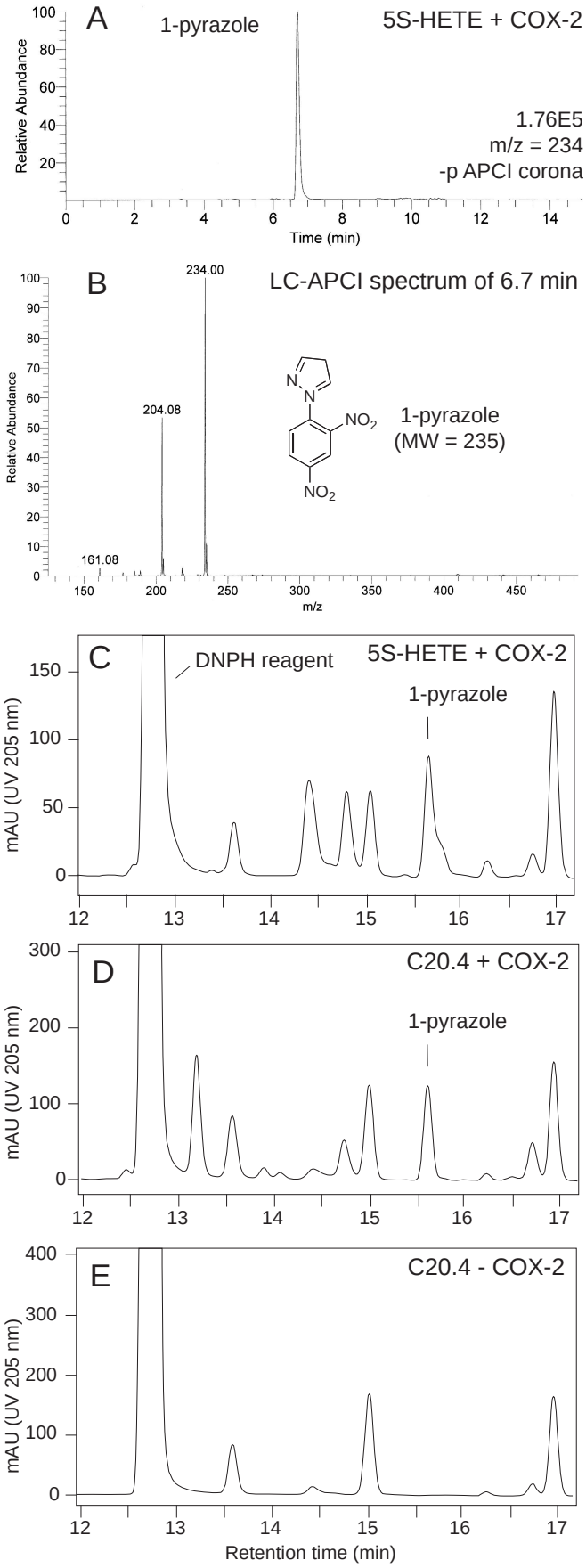

A
1-pyrazole
5S-HETE + COX-2
1.76E5
m/z = 234
-p APCI corona
Relative Abundance
Time (min)
B
LC-APCI spectrum of 6.7 min
234.00
204.08
161.08
1-pyrazole
(MW = 235)
m/z
C
DNPH reagent
5S-HETE + COX-2
1-pyrazole
mAU (UV 205 nm)
D
C20.4 + COX-2
1-pyrazole
E
C20.4 - COX-2
Retention time (min)

Fig. 4

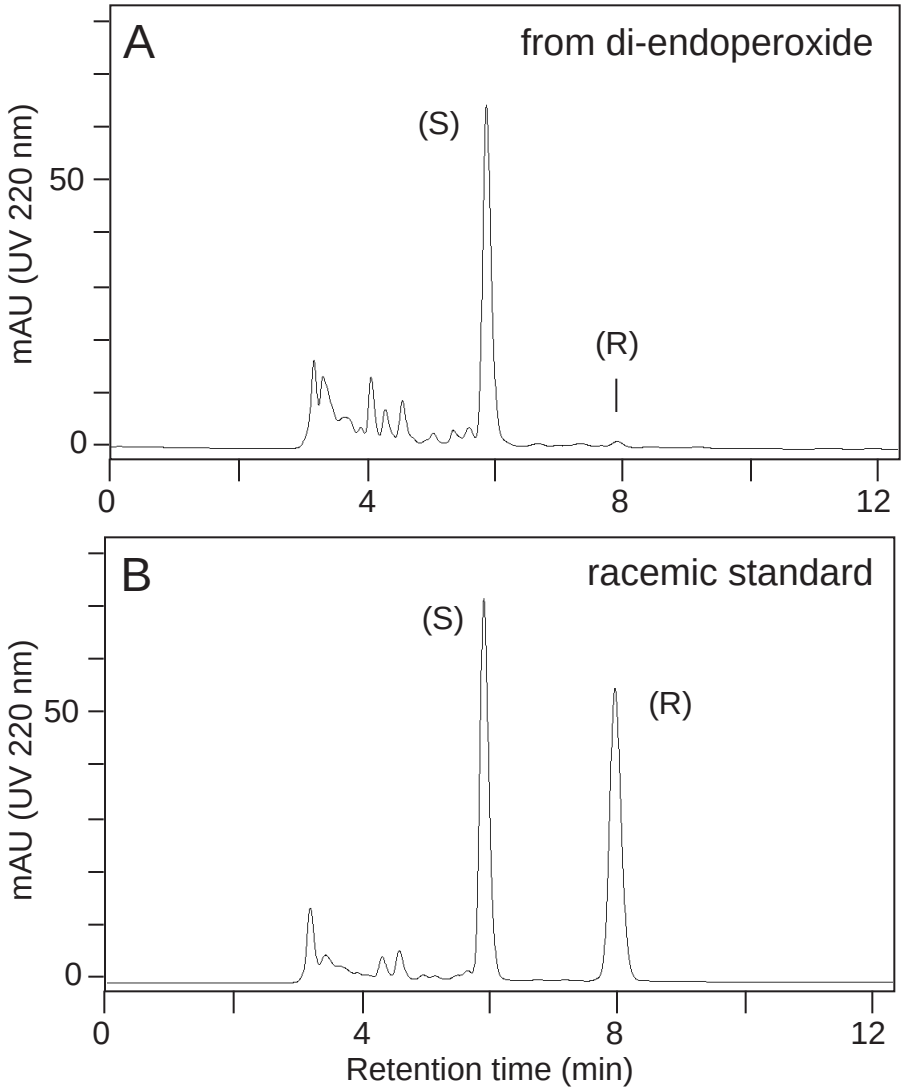
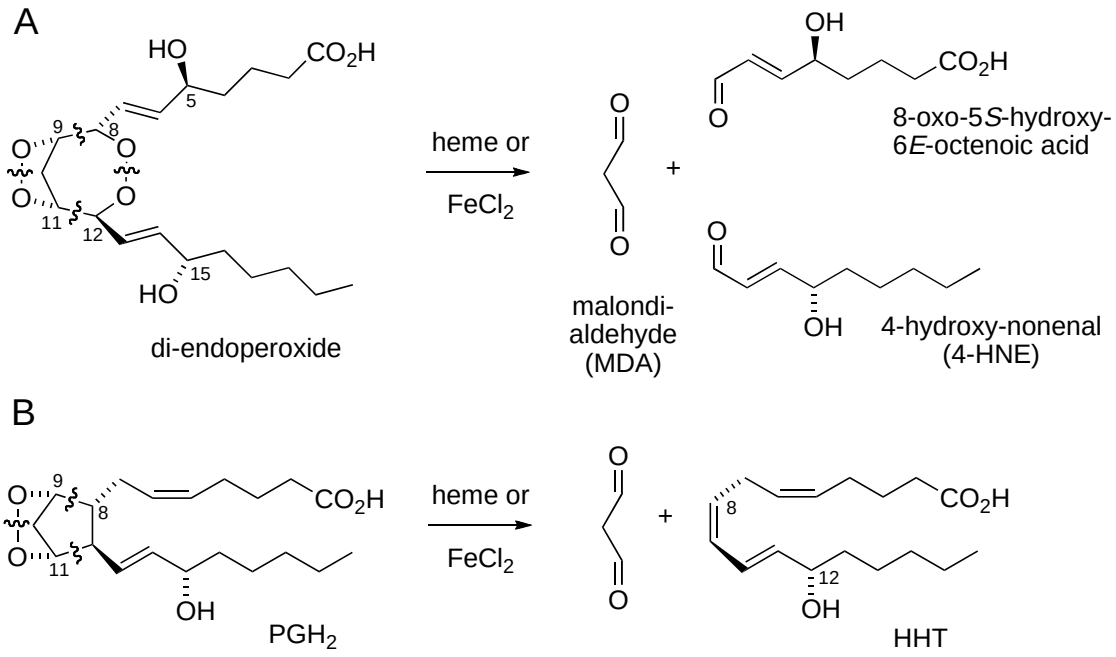

A
from di-endoperoxide
(S)
(R)
mAU (UV 220 nm)
50
0
0
4
8
12
B
racemic standard
(S)
(R)
mAU (UV 220 nm)
50
0
0
4
8
12
Retention time (min)

Fig. 5


A
HO
CO2H
5
9
8
O
O
O
O
11
12
15
HO
di-endoperoxide
heme or
FeCl2
O
O
malondi-
aldehyde
(MDA)
+
OH
CO2H
O
8-oxo-5S-hydroxy-
6E-octenoic acid
O
OH
4-hydroxy-nonenal
(4-HNE)
B
9
O
8
CO2H
O
11
OH
PGH2
heme or
FeCl2
O
O
+
8
CO2H
12
OH
HHT

Identification and absolute configuration of dihydroxy-arachidonic acids formed by oxygenation of 5*S*-hydroxyeicosatetraenoic acid by native and aspirin-acetylated cyclooxygenase-2

Surafel Mulugeta, Takashi Suzuki, Noemi Tejera Hernandez, Markus Griesser[1], William E. Boeglin, and Claus Schneider

Division of Clinical Pharmacology, Department of Pharmacology, Vanderbilt University Medical School, Nashville, Tennessee 37232, U.S.A.

[1] present address: Consumer Safety, Crop Protection, BASF SE, Germany

correspondence address:
Claus Schneider, Ph.D., Division of Clinical Pharmacology, Department of Pharmacology, Vanderbilt University Medical School, RRB 572, 23rd Ave. S. at Pierce, Nashville, TN 37232-6602, U.S.A., Tel.: 615-343-9539, Fax: 615-322-4707, email: claus.schneider@vanderbilt.edu

Running footline: DiHETEs from cross-over of the 5-LOX and COX-2 pathways

Abbreviations used:

ATL	aspirin-triggered lipoxin
CD	circular dichroism
CE	Cotton effect
CID	collision induced dissociation
COX-2	cyclooxygenase-2
diHETE	dihydroxy-eicosatetraenoic acid
HETE	hydroxy-eicosatetraenoic acid
5-LOX	5-lipoxygenase
LPS	lipopolysaccharide
PG	prostaglandin
SRM	selected reaction monitoring
TPP	triphenylphosphine

Abstract

Biosynthesis of the prostaglandin endoperoxide by the cyclooxygenase (COX) enzymes is accompanied by formation of a small amount of 11*R*-hydroxyeicosatetraenoic acid (11*R*-HETE), 15*R*-HETE, and 15*S*-HETE as by-products. Acetylation of COX-2 by aspirin abrogates prostaglandin synthesis and triggers formation of 15*R*-HETE as the sole product of oxygenation of arachidonic acid. Here we investigated the formation of by-products of the transformation of 5*S*-HETE by native COX-2 and by aspirin-acetylated COX-2 using HPLC-UV, GC-MS, and LC-MS analysis. 5*S*,15*S*-diHETE, 5*S*,15*R*-diHETE, and 5*S*,11*R*-diHETE were identified as by-products of native COX-2, in addition to the di-endoperoxide as the major oxygenation product. 5*S*,15*R*-diHETE was the only product formed by aspirin-acetylated COX-2. Both 5,15-diHETE and 5,11-diHETE were detected in CT26 mouse colon carcinoma cells as well as in LPS-activated RAW264.7 cells incubated with 5*S*-HETE, and their formation was attenuated in the presence of the COX-2 specific inhibitor, NS-398. Aspirin-treated CT26 cells gave 5,15-diHETE as the most prominent product formed from 5*S*-HETE. 5*S*,15*S*-diHETE has been described as a product of the cross-over of 5-lipoxygenase (5-LOX) and 15-LOX activities in elicited rat mononuclear cells and human leukocytes, and our studies implicate cross-over of 5-LOX and COX-2 pathways as an additional biosynthetic route.

Supplementary key words: lipoxygenase, 5-LOX, diHETE, arachidonic acid, macrophage, LC-MS, circular dichroism

Introduction

Oxygenation of arachidonic acid by either of the two cyclooxygenase (COX) isozymes yields the prostaglandin endoperoxide PGH_2 as the major product and the mono-hydroxylated 11-HETE and 15-HETE as by-products of about 2-5% abundance (1). 11-HETE is exclusively of the 11*R* configuration, similar in configuration to the first oxygenation of arachidonic acid to the 11*R*-peroxyl radical that will form the 9,11-endoperoxide of PGH_2 (2, 3). 15-HETE is formed as a mixture of the 15*S*- and 15*R*-enantiomers, in contrast to the configuration of C15 in PGH_2 which is strictly *S* (4). The HETE by-products are thought to arise from a slightly different alignment of substrate in the active site compared to when PGH_2 is formed (5), rather than resulting from incomplete or otherwise faulty catalysis. Neither 15-HPETE nor 11-HPETE can serve as substrates for formation of PGH_2 in the cyclooxygenase reaction as demonstrated for the COX-1 enzyme (6, 7). It has not been established whether formation of the HETE by-products follows a particular biological rationale.

Acetylation by aspirin (acetylsalicylic acid) of a serine residue in the oxygenase active site channel of both COX isozymes has discrete effects on the catalytic activities of the two enzymes (8, 9). Whereas COX-1 loses all oxygenase activity following treatment with aspirin, acetylated COX-2 gains a novel catalytic activity and forms 15*R*-HETE as the sole product (10, 11).

The major product formed by oxygenation of the 5-lipoxygenase product, 5*S*-HETE, with COX-2 is a bicyclic di-endoperoxide with structural similarities to the arachidonic acid derived PGH_2 (12). The most significant difference between the two endoperoxides is that the typical cyclopentyl ring of PGH_2, comprised of carbons 8 through 12, is extended to a seven-membered ring by insertion of a peroxide bridge from C8 to C12 in the 5-HETE derived di-endoperoxide. In addition, the di-endoperoxide contains two hydroxy groups, one at carbon 5 stemming from the 5*S*-HETE substrate, and the other at C15, equivalent to the 15-hydroxy in the prostaglandins. The relative and absolute

stereochemistries of carbons 9, 11, and 15 are the same in PGH_2 and the di-endoperoxide, i.e., 9*S*, 11*R*, and 15*S* (13).

Here we report the structural identification and absolute configuration of two by-products of the COX-2 reaction with 5*S*-HETE. In addition, we analyzed the reaction of acetylated recombinant COX-2 with 5*S*-HETE. Finally, formation of diHETEs from exogenous 5*S*-HETE was confirmed to be dependent on COX-2 in two mouse cell lines, RAW264.7 and CT26.

Experimental Procedures

Materials

Arachidonic acid was purchased from NuChek Prep. Inc. (Elysian, MN), LPS (serotype 0111:B4) was from Calbiochem, and RAW264.7 and CT26 cells were obtained from ATCC (Manassas, VA). 5*S*-HETE was prepared by chemical synthesis from arachidonic acid as described (13). 15*R*-HETE and 11*R*-HETE were prepared through vitamin E-controlled autoxidation of arachidonic acid methyl ester and purified by consecutive RP-, SP-, and chiral phase HPLC (Chiralpak AD (14)), and a final step of mild hydrolysis of the methyl ester using KOH.

Cell culture

RAW264.7 cells were cultured in DMEM and grown at 37°C in an atmosphere of 5% CO_2. Cells of passages 5 and 6 only were used. Cells were stimulated by treatment with 100 ng/ml LPS and 10 units/ml of IFN-γ for 6 h to induce expression of COX-2. CT26 cells were cultured in RPMI 1640 medium. 5*S*-HETE, 5 μg dissolved in 1 μl of ethanol, was added to ≈70 % confluent cells in 100 mm dishes, and after 10 min at 37°C the culture medium was removed, acidified to pH 4, and extracted using a 30-mg Waters HLB cartridge. Products were eluted from the cartridge with methanol, evaporated, and dissolved in 50 μl of LC-MS solvent A. CT26 and RAW264.7 control cells were not treated with LPS. In some experiments, CT26 and activated RAW264.7 cells were treated with 2 mM aspirin, respectively (from a 40 mM stock solution in DMSO), or with 10 μM NS-398 (from a 10 mM stock solution in ethanol) 30 min prior to incubation with 5*S*-HETE.

Reaction of recombinant COX-2 with 5S-HETE

The reaction of 5*S*-HETE (120 μg total; containing 300,000 cpm of [1-^{14}C]5*S*-HETE) with recombinant human COX-2 was performed in 4 separate 2 ml-reactions with 30 μg substrate each as described (12). The products were extracted using a Waters HLB cartridge and analyzed by RP-HPLC using a Waters Symmetry C18 5-μm column (4.6 x 250 mm) eluted with a gradient of acetonitrile/water/acetic acid programmed from

20/80/0.01 (by vol.) to 70/30/0.01 (by vol.) within 20 min at 1 ml/min flow rate. The elution profile was monitored using an Agilent 1200 diode array detector coupled on-line to a Packard Radiomatic A100 Flo-one radioactive detector. The by-products eluting at 19.8 and 20.5 min retention time were collected, extracted from HPLC solvent, and stored in methanol at -20°C until further analysis.

Reaction of aspirin-acetylated COX-2 with 5S-HETE

Recombinant human COX-2 (0.5 μM final concentration) was diluted in 1 ml of 100 mM Tris-HCl buffer pH 8.0 and treated with 2 mM aspirin in a 37°C water bath for 30 min (15). A control reaction incubated with arachidonic acid and analyzed by LC-MS before and after treatment showed greater than 95% inhibition of PG formation. The buffer was supplemented with hematin (1 μM) and phenol (500 μM), and 30 μg of 5*S*-HETE were added. After 5 min at room temperature, 25 μl of methanol were added, the mixture was acidified to pH 4 with glacial acetic acid, and loaded onto a preconditioned Waters Oasis HLB cartridge. After washing with water the products were eluted with methanol. The 5,15-diHETE product was isolated using RP-HPLC as described above for the native enzyme.

Synthesis and isolation of diHETE reference compounds

5*S*,15*S*-DiHETE was synthesized by reaction of 5*S*-HETE with the LOX-1 isozyme from soybean seeds (16). To 3 ml of 100 mM K_2HPO_4 buffer pH 10 were added 100 μg of 5*S*-HETE and 1 μl of soybean lipoxygenase solution (Sigma). 5*R*,15*S*-DiHETE was synthesized using 5*R*-HETE (100 μg) as substrate and 3 μl of soybean lipoxygenase solution. After 1 min reaction time, the solution was acidified (pH 4) and extracted with methylene chloride. The organic extract was evaporated, dissolved in methanol, and treated with 200 μg of triphenylphosphine for 15 min at room temperature. 5*S*,15*S*-DiHETE and 5*R*,15*S*-diHETE were isolated by RP-HPLC using a Waters Symmetry C18 column (4.6 x 250 mm) eluted with a solvent of methanol/water/acetic acid (80/20/0.01, by vol.) at 1 ml/min flow rate and UV detection at 235 nm.

5*S*,15*R*-DiHETE was synthesized by reaction of 15*R*-HETE with recombinant human 5-LOX. For the enzymatic transformation a pellet of *Sf9* insect cells expressing 5-LOX (≈300 µl) was sonicated and transferred to 1 ml of PBS containing 2 mM $CaCl_2$ and 1 mM ATP. 15*R*-HETE (50 µg) was added and the reaction was allowed to proceed for 15 min at room temperature. The reaction was terminated by the addition of 250 µl of methanol and 10 mg of $NaBH_4$. After 15 min at room temperature, the pH was adjusted to 3 using 1 N HCl, and the products were extracted using methylene chloride. 5*S*,15*R*-DiHETE was purified by RP-HPLC as described above for the 5*S*,15*S*-diastereomer.

5*S*,11*R*-DiHETE was synthesized by reaction of 5*S*-HETE with the recombinant linoleic acid 9*R*-LOX from *Anabaena* sp. PCC7120 expressed in *E. coli*, a gift from Alan R. Brash at Vanderbilt University (17). The substrate, 5*S*-HETE (100 µg), was added to 3 ml of 100 mM Tris-HCl pH 7.5 containing 150 mM NaCl and 0.01 % CHAPS. The reaction was initiated by addition of 1 µl of the purified *Anabaena* 9*R*-LOX. After 3 min reaction time, the solution was acidified to pH 4 using 1 N HCl, and the products were extracted using a 30-mg Waters HLB cartridge and eluted with methanol. After evaporation of the solvent, the residue was dissolved in 100 µl of methanol, and the products were reduced with 150 µg TPP at room temperature for 15 min. 5*S*,11*R*-DiHETE was isolated using RP-HPLC conditions as described above for 5*S*,15*S*-diHETE. HPLC-purified 5*S*,11*R*-diHETE was dissolved in $CDCl_3$ for NMR analysis using a Bruker AV-II 600 MHz spectrometer equipped with a cryoprobe. Chemical shifts are reported relative to the signal for residual $CHCl_3$ at δ 7.25 ppm.

A mixture of 5,11-diHETE diastereomers was synthesized by autoxidation of racemic 11-HETE. Three 200 µg aliquots of 11-HETE were evaporated in small plastic tubes and placed in an oven at 37°C. After 2 h the samples were dissolved in 50 µl of methanol, treated with TPP and analyzed using RP-HPLC. The diastereomers eluted as a single peak, and purification was performed as described for 5*S*,15*S*-diHETE.

SP-HPLC analysis of diHETEs

The 5,15-diHETE diastereomers were resolved using an Agilent Zorbax RX-SIL 5-μm column (4.6 x 250 mm) eluted with hexane/isopropanol/acetic acid (95/5/0.1, by vol.) at 1 ml/min flow rate. The 5,11-diHETEs were analyzed using the same HPLC conditions after conversion to the methyl ester derivatives with diazomethane. Eluting peaks were monitored using an Agilent 1200 series diode array detector.

CD spectroscopy

Aliquots of about 20 μg each of 5*S*-HETE, 15*S*-HETE, 15*R*-HETE, 11*S*-HETE, 11*R*-HETE, and the enzymatically synthesized standards of 5*S*,15*S*-diHETE, 5*R*,15*S*-diHETE, and 5*S*,11*R*-diHETE were treated with ethereal diazomethane for 30 s, evaporated, and dissolved in 50 μl of dry acetonitrile. To the solution was added 1 μl of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), and a few grains each of 4-dimethylaminopyridine (DMAP) and 2-naphthoylchloride. The reaction was carried out at room temperature overnight, the solvent was evaporated, and the residue was dissolved in methylene chloride and washed with water twice. Purification of the methyl ester, 2-naphthoyl derivatives of the HETEs and diHETEs was achieved by RP-HPLC using a Waters Symmetry C18 column (4.6 x 250 mm) eluted with a solvent of methanol/water/acetic acid (95/5/0.01, by vol.) at 1 ml/min flow rate and UV detection at 235 nm. Samples were extracted from HPLC solvent using methylene chloride and dissolved in acetonitrile to an OD of 0.75 AU for HETE derivatives and OD 1.5 AU for diHETE derivatives (when possible), respectively. CD spectra were recorded using an Aviv Model 215 CD spectrometer at room temperature in a 1-cm pathlength cuvette scanning from 350 – 200 nm. The ^{1}H NMR spectrum (600 MHz) of the 2-naphthoate derivatized 5*S*,11*R*-diHETE methyl ester was recorded in CD_3CN, δ 1.93 ppm.

GC-MS and LC-MS analysis

For GC-MS analysis, the 5,15-diHETE and 5,11-diHETE formed yb reaction of COX-2 with 5*S*-HETE were purified using RP- and SP-HPLC, and methylated using ethereal diazomethane. Hydrogenation was performed in 100 μl of ethanol in the presence of Pd/C and bubbling with hydrogen gas for 5 min. Trimethylsilyl ethers were prepared using *bis*(trimethylsilyl)trifluoroacetamide at room temperature for 1 h. The reagents

were evaporated and the samples were dissolved in hexane. GC-MS analysis was carried out in the EI mode (70 eV) using a ThermoFinnigan DSQ mass spectrometer equipped with a 5 m SPB-1 column (0.1 mm i.d., film thickness 0.25 μm) and a temperature program from 100°C, hold 2 min, and then increased to 260°C at 20°C/min.

LC-MS was performed using a ThermoFinnigan Quantum Access instrument equipped with an electrospray interface and operated in the negative ion mode. User modified parameters of sheath and auxiliary gas pressures, temperature, and voltage settings were optimized using direct infusion of a solution of PGD_2. A Waters Symmetry Shield C18 3.5 μm-column (2.1 x 150 mm) was eluted with a linear gradient of acetonitrile/water, 10 mM NH_4OAc (5/95, by vol.; solvent A) to acetonitrile/water, 10 mM NH_4OAc (95/5, by vol.) at a flow rate of 0.2 ml/min within 10 min. Negative ion CID mass spectra of the standards of PGD_2, 5*S*-HETE, 5*S*,15*S*-diHETE, and 5*S*,11*R*-diHETE were obtained. The fragmentation patterns were used to establish ion transitions for analyses in the SRM mode. The following transitions were monitored: for PGD_2 and PGE_2: *m/z* 351 -> 271; 5-HETE: *m/z* 319 -> 115; 5,15-diHETE: *m/z* 335 -> 201; and 5,11-diHETE: *m/z* 335 -> 183. Relative levels of prostaglandins and diHETEs between treatments were calculated using peak areas of the signals in the SRM chromatograms.

Results

Reaction of native and acetylated COX-2 with 5S-HETE

RP-HPLC analysis of the transformation of [1-^{14}C]5*S*-HETE by recombinant COX-2 shows one main product that was identified previously as a highly oxygenated di-endoperoxide (12), in addition to two minor, less polar peaks designated I and II representing by-products of the reaction (Fig. 1A). When COX-2 was treated with aspirin prior to incubation with 5*S*-HETE, one major product (III) was formed with retention time similar to peak I in the untreated enzyme (Fig. 1B). Both I and III had a characteristic UV spectrum with a λmax at 243 nm that was readily identified as 5,15-diHETE (Fig. 1C) (18). The UV spectrum of peak II had a maximum at 238 nm with shoulders around 228 nm and 247 nm (Fig. 1C). The retention time and UV spectrum of II implicated that the product also contained two hydroxy groups and conjugated diene moieties.

The products I and II were isolated using RP-HPLC and further purified as the methyl ester derivatives using SP-HPLC. GC-MS analysis in the EI mode (70 eV) of the hydrogenated, TMS-ether derivatives confirmed the identification of the first peak I as 5,15-diHETE. Characteristic α-cleavage fragments were found at *m/z* 203 (55% relative intensity) and *m/z* 311 (after loss of OTMS; 9%) for the 5-hydroxy, and at *m/z* 173 and 341 (after loss of OTMS) (56% and 7%, respectively) for the 15-hydroxy group; the base peak was at *m/z* 73. Peak III from the aspirin-acetylated COX-2 reaction was identified as 5,15-diHETE based on identical UV spectra and retention times on RP-HPLC, and in addition to subsequent experimental evidence as described below.

Product II gave a very weak [M^+] (*m/z* 502) and [$M\text{-}CH_3^+$] (*m/z* 487) ion, with characteristic α-cleavage fragments at *m/z* 203 (42%) and *m/z* 311 (after loss of OTMS; 4%) indicating a 5-hydroxy group, and at *m/z* 229 (38% relative intensity) and *m/z* 285 (after loss of OTMS) (5%) indicative of a 11-hydroxy group. The LC-ESI mass spectrum confirmed the molecular weight as 336 and also gave a major fragment at *m/z* 183 and a

minor fragment at *m/z* 115, compatible with two hydroxyls at carbons 5 and 11 (Fig. 1D). Based on UV, GC-MS, and LC-MS analyses, product II was identified as 5,11-diHETE.

^{1}H NMR and H,H COSY data for product II were recorded using a chromatographically and spectroscopically (UV, LC-MS/MS) identical standard of 5*S*,11*R*-diHETE that was prepared as described below. The ^{1}H NMR spectrum showed 8 signals in the double bond region that appeared as a pair of two similar motives of 4 protons each comprised of the two conjugated *cis*,*trans*-dienes (H7: δ 6.57 ppm, dd, *J* = 15.1 Hz/11.0 Hz; H8: δ 6.13, dd, *J* = 11.0; H6: δ 5.70, dd, *J* = 14.9 Hz/6.3 Hz, H9: δ 5.55, m; and H13: δ 6.51, dd, *J* = 14.9 Hz/11.4 Hz; H14: 5.96, dd, *J* = 11.0 Hz; H12: δ 5.67, m; H15: δ 5.46, m). Two protons attached to carbons bearing a hydroxyl group were located at 4.25 ppm (H11: δ 4.25, dt, *J* = 6.3 Hz/6.1 Hz) and 4.17 ppm (H5: δ 4.17, dt, *J* = 6.2 Hz/6.0Hz). H4 was detected as a cross-peak from H5 in the H,H-COSY spectrum at 1.57 ppm, H3 was a multiplet (1.70 ppm) and was coupled to the triplet signal of H2 at 2.34 ppm (*J* = 7.4 Hz). Both protons of H10 were detected as a multiplet at 2.47 ppm, and H16 was a dt signal at 2.17 ppm (*J* = 7.6 Hz/7.2 Hz).

The configuration of C-15 in the 5,15-diHETE products (I and III) and of C-11 in the 5,11-diHETE (II) was established by co-elution with corresponding diHETE diastereomers of known configuration. The configuration of the 5-hydroxy group in all diHETE products was expected to be unchanged from the starting substrate, 5*S*-HETE.

Synthesis of standards of diastereomeric diHETEs

Table 1 gives an overview of the diHETE standards prepared as reference compounds. Authentic 5*S*,15*S*-diHETE was prepared by reaction of soybean LOX-1 with 5*S*-HETE. Synthesis of 5*S*,15*R*-diHETE by reaction of 15*R*-HETE with the recombinant human 5-LOX gave only a minor yield of product, albeit it was sufficient to determine the retention times on RP- and SP-HPLC. In addition, the enantiomer 5*R*,15*S*-diHETE was prepared by reaction of 5*R*-HETE with the lipoxygenase from soybean seeds. 5*S*,15*R*-diHETE and 5*R*,15*S*-diHETE have indistinguishable retention times on RP- and SP-HPLC.

An authentic standard of 5*S*,11*R*-diHETE was prepared by reaction of 5*S*-HETE with the recombinant 9*R*-LOX from *Anabaena* sp PCC7120. A mixture of the 5,11-diHETE diastereomers was prepared by thin-film autoxidation of racemic 11-HETE. Initial attempts to prepare 5*S*,11*S*- and 5*S*,11*R*-diHETEs by reaction of 11*S*-HETE and 11*R*-HETE, respectively, with the recombinant human 5-LOX did not yield a significant amount of either 5,11-diHETE diastereomer. The assignment of the absolute configuration of the hydroxy groups in the diHETE standards was confirmed using CD spectroscopy (see below).

Absolute configuration of 5,15-diHETEs from native and acetylated COX-2

The diastereomers of 5,15-diHETE do not resolve on RP-HPLC (18), but there is adequate separation on SP-HPLC to allow for secure assignment of the absolute configuration at C-15. Therefore, the 5,15-diHETEs were first isolated as a single peak using RP-HPLC and then resolved using SP-HPLC (Fig. 2). The 5,15-diHETE (peak I) isolated form the reaction of human COX-2 gave a %-ratio for 5*S*,15*S*-diHETE to 5*S*,15*R*-diHETE of 77:33, 80:20, and 75:25 in three separate experiments (Fig. 2A). The authentic standards of 5*S*,15*S*-diHETE and 5*S*,15*R*-diHETE eluted at 12.3 min and 12.8 min retention times, respectively (Fig. 2B, 2C). Peak identification was further confirmed by co-chromatography with the authentic standards (Fig. 2D). Aspirin-treatment of human COX-2 resulted in a shift of the chiral distribution of 5,15-diHETE, and now the product (peak III) was 95% 5*S*,15*R*-diHETE (Fig. 3).

Absolute configuration of 5,11-diHETEs

Standards for the 5*S*,11*R*- and 5*S*,11*S*-diHETE diastereomers were prepared by thin film autoxidation of racemic 11-HETE followed by reduction with triphenylphosphine. 5,11-DiHETE was the almost exclusive diHETE formed, and the diastereomers eluted as a single peak when analyzed by RP-HPLC. Using SP-HPLC satisfactory resolution of the methyl ester derivatives was achieved (Fig. 4). The first peak comprised of the 5*S*,11*S*- and 5*R*,11*R*-diastereomers eluted at 17.8 min and the second peak (5*S*,11*R*- and 5*R*,11*S*-diastereomers) eluted at 18.3 min. The authentic standard of 5*S*,11*R*-diHETE prepared

using the *Anabaena* LOX co-eluted with the second peak on SP-HPLC and established the elution order. SP-HPLC analysis of 5,11-diHETE from recombinant human COX-2 showed that the configuration was greater than 98% 5*S*,11*R*-diHETE.

CD-spectroscopy of diHETE standards

We used the exciton-coupled chiral dichroism method in order to confirm assignment of the absolute configuration of the hydroxy groups in the diHETE standards. This method uses the coupling of two chromophores attached to the chiral center in circular polarized light in order to determine the absolute configuration from the sign of the Cotton effects. One of the chromophores in the (di)HETEs is present as the conjugated diene system, and in order to introduce the second chromophore the hydroxy groups were derivatized to the 2-naphthoate ester (19). Mono- and di-2-naphthoate derivatives, respectively, of the following methyl ester fatty acids were prepared: 5*S*-HETE, 11*S*-HETE, 11*R*-HETE, 15*S*-HETE, 15*R*-HETE, 5*S*,15*S*-diHETE, 5*R*,15*S*-diHETE, and 5*S*,11*R*-diHETE.

As expected, pairs of derivatized enantiomers, e.g., 15*S*-HETE and 15*R*-HETE, gave mirror-image CD spectra (Fig. 5). Furthermore, the CD spectra of all *S*-configuration HETEs (5*S*-HETE, 11*S*-HETE, and 15*S*-HETE) gave a positive first CE (i.e., the CE at the higher wavelength) and a negative second CE. Due to the additive nature of the absorbance in the CD spectrum, the two chiral centers in the diHETEs were expected to result in a CD spectrum that represents the mathematical sum of the spectra obtained for the two individual chiral centers. The spectrum of 5*S*,15*S*-diHETE showed increased intensities for the two CEs, although the Δε intensities were not doubled when compared to 15*S*-HETE which was likely due to saturation effects at the high concentration measured (1.5 AU in the UV) (Fig. 5). In contrast, for the 5*R*,15*S*-diHETE diastereomer the CEs cancelled each other out and the resulting CD spectrum was an almost flat line. The CD spectra of the diastereomeric 5,15-diHETEs confirmed the assignment of the absolute configuration of the two chiral centers in the 5*S*,15*S*-diHETE standard.

The configuration of C11 in 5*S*,11*R*-diHETE formed by reaction of the *Anabaena* 9-LOX with 5*S*-HETE was confirmed using the same approach. The individual CD spectra of 2-

naphthoate-derivatized 5*S*-HETE and 11*R*-HETE are mirror images of each other, but unexpectedly, the CD spectrum of 5*S*,11*R*-diHETE was not a flat line (Fig. 6). The spectrum showed CEs at 245 nm (Δε +7.6) and 228 nm (Δε -6.7), resembling a weaker version of the CD spectrum of 5*S*-HETE with slightly shifted maxima. We hypothesized that the transition moment of the chromophores at C5 gave a stronger spectrum due to more optimal alignment of the two chromophores, thereby overcompensating the spectrum for the chiral center at C11. ^{1}H NMR determination of the $J_{5,6}$ and $J_{11,12}$ coupling constants that can be taken as a measure for the alignment of the chromophores within the conformer gave essentially equivalent values, i.e, 6.7 Hz and 6.8 Hz, respectively. Since SP-HPLC has confirmed the relative configuration of the 5*S*,11*R*-diHETE (Fig. 4D), the question why the corresponding CD spectrum showed slight predominance of the *S*-configurated chiral center remains unexplained.

Formation of diHETEs in RAW264.7 and CT26 cells

RAW264.7 were treated in four different ways and incubated with 4 μM 5*S*-HETE. We used non-stimulated cells, cells stimulated with LPS only, and LPS-stimulated cells treated with NS-398 or aspirin, respectively. Formation of diHETEs was analyzed using negative ion LC-ESI-MS in the SRM mode (Fig. 7A). Both 5,15-diHETE and 5,11-diHETE were detected in RAW264.7 cells activated with LPS and IFN-γ (Fig. 7A, upper panel), and their concentration was reduced to 0.5% and 3%, respectively, by incubation of the cells with the COX-2 inhibitor NS-398 (10 μM) prior to the addition of 5*S*-HETE (Fig. 7A, middle panel). Treatment of RAW264.7 cells with 2 mM aspirin led only to a 30% reduction in the level of PGD_2, and about 60% and 50% reduction in 5,15-diHETE and 5,11-diHETE, respectively (Fig. 7A, lower panel). In the RAW264.7 cells aspirin showed only little efficacy in affecting eicosanoid formation, consistent with the findings reported by (20).

Formation of diHETEs was also analyzed in CT26 mouse colon carcinoma cells incubated with 5*S*-HETE. CT26 cells incubated with 5*S*-HETE showed robust formation of 5,15-diHETE and 5,11-diHETE, and the levels of both were reduced >100-fold by pre-incubation with 10 μM NS-398 (Fig. 7B). Pre-treatment with 2 mM aspirin inhibited the

formation of 5,11-diHETE by about 90%, and 5,15-diHETE was reduced to only 23% compared to the cells not treated with aspirin, reflecting enhanced formation of 5*S*,15*R*-diHETE.

Discussion

After the initial oxygenation of arachidonic acid to form a conjugated diene hydro(pero)xide (H(P)ETE) additional sites remain in the molecule for subsequent reaction with molecular oxygen (21). This opens the possibility for formation of di-hydroxylated (diHETEs) and tri-hydroxylated derivatives of arachidonic acid. Enzymatic synthesis of diHETEs can be catalyzed via several distinct routes, all of which involve one or more LOX reactions: (i) The consecutive reaction of two separate LOX enzymes is involved in the biosynthesis of 5*S*,15*S*-diHETE in elicited rat mononuclear cells and human leukocytes (18). In this case, arachidonic acid is first oxygenated by 5-LOX followed by 15-LOX or *vice versa*. (ii) An alternative route to 5*S*,15*S*-diHETE is through double oxygenation of arachidonic acid catalyzed by a single LOX enzyme. This possibility is best recognized for the LOX-1 isozyme from soybean seeds in the formation of 5*S*,15*S*-diH(P)ETE and 5*S*,8*S*-diH(P)ETE by oxygenation of the primary product 15*S*-H(P)ETE (22, 23). (iii) The third possibility is exemplified by the biosynthesis of LTB_4 and 12-*epi*-LTB_4 (i.e., 5*S*,12*R*-diHETE and 5*S*,12*S*-diHETE, respectively). In this case, the diHETEs are hydrolysis products of the unstable LTA_4 epoxide. The LTA_4 epoxide is formed by 5-LOX catalyzing a second hydrogen abstraction (at C-10) of its initial 5*S*-HPETE product. But rather than inserting a second molecule of oxygen the reaction is completed by dehydration of the existing hydroperoxide to give the epoxide (24, 25). And lastly (iv), a fourth distinct route to diHETEs is implicated by the findings presented in this report. This route involves a cross-over of the activities of the 5-LOX and COX-2 enzymes, with the 5-LOX product 5*S*-HETE being oxygenated by COX-2 to form – as by-products – a mixture of 5*S*,15*S*-diHETE, 5*S*,15*R*-diHETE, and 5*S*,11*R*-diHETE.

The formation of 5,15-diHETE and 5,11-diHETE as by-products of the COX-2 catalyzed transformation of 5*S*-HETE bears strong resemblance of the formation of 15-HETE and 11-HETE as by-products of the COX-catalyzed transformation of arachidonic acid to PGH_2 (Fig. 8) (5, 26). In contrast to the reaction with arachidonic acid, 5*S*-HETE reacts only with COX-2; the COX-1 isozyme is inactive with 5*S*-HETE (12). The configuration

of C-15 of 5,15-diHETE was a ≈3.5:1 mixture of 15*S* and 15*R*, and a similar mixture of 15*S* and 15*R* configuration is found in the 15-HETE formed by COX-1 and COX-2 (4). The configuration of C-11 in 5,11-diHETE is >98% 11*R*, identical to the near exclusive 11*R*-configuration of 11-HETE formed by COX-1 and COX-2 (2, 3, 27). We can conclude that the modes of binding of arachidonic acid and of 5*S*-HETE in the cyclooxygenase active site must be very similar. There is precise control over the C-11 and C-15 oxygenations in the formation of the prostaglandin endoperoxide and the di-endoperoxide as well as in the formation of the 11*R*-HETE and 5*S*,11*R*-diHETE by-products, respectively (28). There is less control of the oxygen insertion and/or less tight binding of the ω-tail of the fatty acid substrate in the case of the formation of the 15-HETE or 5,15-diHETE by-products. The major difference in catalytic outcome with 5*S*-HETE, however, is the insertion of another molecule of oxygen in place of the C8-C12 carbon bond resulting in the formation of two endoperoxide rings.

Acetylation of Ser-516 in the COX-2 active site by aspirin (acetylsalicylic acid) has a remarkable effect on its catalytic activity (10, 11). Formation of the prostaglandin endoperoxide is prevented, and instead a novel catalytic activity is gained forming 15*R*-HETE as the sole enzymatic product. The basis for the complete inversion of the stereochemistry of C15 from 15*S* in PGH_2 to 15*R* in 15*R*-HETE has not been definitely elucidated but it likely involves a change in the binding of the ω-end of arachidonic acid beyond C-13 in the channel above Ser-516 (28-32). When 5*S*-HETE was incubated with acetylated COX-2, formation of the di-endoperoxide was inhibited, and 5,15-diHETE was the only product detected. Not too surprisingly, the configuration of C-15 of the 5,15-diHETE was found to be >95% 15*R*.

The formation of diHETEs was analyzed using the RAW264.7 mouse macrophage cells and CT27 mouse colon carcinoma cells. 5,15-DiHETE and 5,11-diHETE were detected upon incubation of both cell types with 5*S*-HETE. Formation of the diHETEs was dependent on COX-2 because they were absent in non-stimulated cells and in cells treated with the COX-2 inhibitor NS-398. Treatment of CT26 cells with aspirin prior to incubation with 5*S*-HETE enhanced the biosynthesis of 5,15-diHETE, consistent with the

findings using recombinant COX-2 enzyme. Aspirin, even at a high concentration, did not show this effect in RAW264.7 cells, most likely due to lesser efficacy for covalent modification of the COX enzyme in cells with a highly oxidative tone (20, 33). 5*S*,11*R*-diHETE and 5*S*,15*R*-diHETE have not been described as metabolites of arachidonic acid before, although there are two reports published more than 20 years ago that implicate the possibility of COX-dependent biosynthesis of diHETEs in human umbilical arteries (34, 35). Unfortunately, the presumed diHETE metabolites were left uncharacterized, and it is difficult to estimate whether the products described could be similar or identical to 5,15-diHETE or 5,11-diHETE.

Our studies invoke the possibility of an alternative route of biosynthesis of 5*S*,15*S*-diHETE, and therefore, additional experiments are required to distinguish whether, if detected *in vivo*, 5,15-diHETE is formed by cross-over of the 5-LOX and 15-LOX pathways, or by cross-over of the 5-LOX and COX-2 pathways. Involvement of COX-2 can be implicated if the formation of 5,15-diHETE is attenuated upon application of a COX-2 specific inhibitor. Alternatively, a minor amount of 5,11-diHETE in addition to 5,15-diHETE could be indicative of COX-2 involvement. Formation of 5*S*,15*R*-diHETE as an alternative specific marker of COX-2 involvement is difficult to establish since the 5*S*,15*S*- and 5*S*,15*R*-diastereomers do not resolve using standard RP-HPLC conditions.

Formation of 5*S*,15*R*-diHETE is somewhat reminiscent of formation of the so-called aspirin-triggered lipoxins (ATLs) (36). Both products involve cross-over of the activities of aspirin-acetylated COX-2 and 5-LOX. ATLs are formed by the reaction of 15*R*-HETE (the metabolite of acetylated COX-2) with the 5-LOX enzyme initially forming the 5*S*-hydroperoxide of 15*R*-HETE followed by dehydration to the 5*S*,6*S*-epoxy-15*R*-hydroxy derivative, analogous to the biosynthesis of the leukotriene epoxide LTA_4. Hydrolysis of the epoxy-tetraene at carbons 6 or 14 affords 5*S*,6*R*,15*R*-trihydroxy-eicosatetraenoic acid (15-*epi*-lipoxin A_4) and 5*S*,14*R*,15*R*-trihydroxy-eicosatetraenoic acid (15-*epi*-lipoxin B_4), respectively (37, 38). In contrast, the enzymatic activities are coupled “the other way round” for formation of 5*S*,15*R*-diHETE, i.e., 5-LOX first produces 5*S*-HETE which is subsequently converted by acetylated COX-2 to 5*S*,15*R*-diHETE.

Acknowledgements

This work was supported by award number R01GM076592 from the National Institutes of General Medical Sciences and from the Department of Defense Breast Cancer Research Program (BC063074). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of General Medical Sciences or the National Institutes of Health. S.M. was supported by the ASPET Summer Undergraduate Research Fellowship. We thank Dr. David Wright and Jonas Perez for assistance in using the CD spectrometer.

References

1. Hamberg, M., and B. Samuelsson. 1967. Oxygenation of unsaturated fatty acids by the vesicular gland of sheep. *J. Biol. Chem.* **242**: 5344-5354.

2. Hawkins, D. J., and A. R. Brash. 1987. Eggs of the sea urchin *Strongylocentrotus purpuratus*, contain a prominent (11R) and (12R) lipoxygenase activity. *J. Biol. Chem.* **262**: 7629-7634.

3. Baer, A. N., P. B. Costello, and F. A. Green. 1991. Stereospecificity of the hydroxyeicosatetraenoic and hydroxyoctadecadienoic acids produced by cultured bovine endothelial cells. *Biochim. Biophys. Acta* **1085**: 45-52.

4. Schneider, C., W. E. Boeglin, J. J. Prusakiewicz, S. W. Rowlinson, L. J. Marnett, N. Samel, and A. R. Brash. 2002. Control of prostaglandin stereochemistry at the 15-carbon by cyclooxygenases-1 and 2. A critical role for serine 530 and valine 349. *J. Biol. Chem.* **277**: 478-485.

5. Thuresson, E. D., K. M. Lakkides, and W. L. Smith. 2000. Different catalytically competent arrangements of arachidonic acid within the cyclooxygenase active site of prostaglandin endoperoxide H synthase-1 lead to the formation of different oxygenated products. *J. Biol. Chem.* **275**: 8501-8507.

6. Hamberg, M., and B. Samuelsson. 1967. On the mechanism of biosynthesis of prostaglandins E_1 and $F_{1\alpha}$. *J. Biol. Chem.* **242**: 5336-5343.

7. Porter, N. A., R. A. Wolf, W. R. Pagels, and L. J. Marnett. 1980. A test for the

intermediacy of 11-hydroperoxyeicosa-5,8,12,14-tetraenoic acid [11-HPETE] in prostaglandin biosynthesis. *Biochem. Biophys. Res. Commun.* **92**: 349-355.

8. DeWitt, D. L., E. A. El-Harith, S. A. Kraemer, M. J. Andrews, E. F. Yao, R. L. Armstrong, and W. L. Smith. 1990. The aspirin and heme-binding sites of ovine and murine prostaglandin endoperoxide synthases. *J. Biol. Chem.* **265**: 5195-5198.

9. Lecomte, M., O. Laneuville, C. Ji, D. L. DeWitt, and W. L. Smith. 1994. Acetylation of human prostaglandin endoperoxide synthase-2 (cyclooxygenase-2) by aspirin. *J. Biol. Chem.* **269**: 13207-13215.

10. Holtzman, M. J., J. Turk, and L. P. Shornick. 1992. Identification of a pharmacologically distinct prostaglandin H synthase in cultured epithelial cells. *J. Biol. Chem* **267**: 21438-21445.

11. Mancini, J. A., G. P. O'Neill, C. Bayly, and P. J. Vickers. 1994. Mutation of serine-516 in human prostaglandin G/H synthase-2 to methionine or aspirin acetylation of this residue stimulates 15-R-HETE synthesis. *FEBS. Lett.* **342**: 33-37.

12. Schneider, C., W. E. Boeglin, H. Yin, D. F. Stec, and M. Voehler. 2006. Convergent oxygenation of arachidonic acid by 5-lipoxygenase and cyclooxygenase-2. *J. Am. Chem. Soc.* **128**: 720-721.

13. Griesser, M., W. E. Boeglin, T. Suzuki, and C. Schneider. 2009. Convergence of the 5-LOX and COX-2 pathways. Heme-catalyzed cleavage of the 5S-HETE-derived di-endoperoxide into aldehyde fragments. *J. Lipid Res.*: in press.

14. Schneider, C., W. E. Boeglin, and A. R. Brash. 2000. Enantiomeric separation of hydroxy-eicosanoids by chiral column chromatography: effect of the alcohol modifier. *Anal. Biochem.* **287**: 186-189.

15. Rowlinson, S. W., B. C. Crews, D. C. Goodwin, C. Schneider, J. K. Gierse, and L. J. Marnett. 2000. Spatial requirements for 15-(*R*)-hydroxy-5*Z*,8*Z*,11*Z*,13*E*-eicosatetraenoic acid synthesis within the cyclooxygenase active site of murine COX-2. Why acetylated COX-1 does not synthesize 15-(*R*)-HETE. *J. Biol. Chem.* **275**: 6586-6591.

16. Brash, A. R., and W.-C. Song. 1996. Detection, assay, and isolation of allene oxide synthase. *Methods Enzymol.* **272**: 250-259.

17. Zheng, Y., W. E. Boeglin, C. Schneider, and A. R. Brash. 2008. A 49-kDa mini-

lipoxygenase from Anabaena sp. PCC 7120 retains catalytically complete functionality. *J Biol Chem* **283**: 5138-5147.

18. Maas, R. L., J. Turk, J. A. Oates, and A. R. Brash. 1982. Formation of a novel dihydroxy acid from arachidonic acid by lipoxygenase-catalyzed double oxygenation in rat mononuclear cells and human leukocytes. *J. Biol. Chem.* **257**: 7056-7067.

19. Schneider, C., P. Schreier, and H.-U. Humpf. 1997. Exciton-coupled circular dichroism (ECCD) in acyclic hydroxylated dienes: a sensitive method for the direct stereochemical assignment of lipoxygenase products. *Chirality* **9**: 563-567.

20. Bala, M., C. N. Chin, A. T. Logan, T. Amin, L. J. Marnett, O. Boutaud, and J. A. Oates. 2008. Acetylation of prostaglandin H2 synthases by aspirin is inhibited by redox cycling of the peroxidase. *Biochem. Pharmacol.* **75**: 1472-1481.

21. Rector, C. L., D. F. Stec, A. R. Brash, and N. A. Porter. 2007. Identification of the peroxidation products of 13-hydroxy-gamma-linolenate and 15-hydroxyarachidonate: mechanistic studies on the formation of leukotriene-like diols. *Chem. Res. Toxicol.* **20**: 1582-1593.

22. Bild, G. S., C. S. Ramadoss, S. Lim, and B. Axelrod. 1977. Double dioxygenation of arachidonic acid by soybean lipoxygenase-1. *Biochem. Biophys. Res. Commun.* **74**: 949-954.

23. Van Os, C. P. A., G. P. M. Rijke-Schilder, H. Van Halbeek, J. Verhagen, and J. F. G. Vliegenthart. 1981. Double dioxygenation of arachidonic acid by soybean lipoxygenase-1. Kinetics and regio-stereo specificities of the reaction steps. *Biochim. Biophys. Acta.* **663**: 177-193.

24. Murphy, R. C., S. Hammarstrom, and B. Samuelsson. 1979. Leukotriene C: a slow-reacting substance from murine mastocytoma cells. *Proc. Natl. Acad. Sci. USA* **76**: 4275-4279.

25. Rouzer, C. A., T. Matsumoto, and B. Samuelsson. 1986. Single protein from human leukocytes possesses 5-lipoxygenase and leukotriene A4 synthase activities. *Proc. Natl. Acad. Sci. USA* **83**: 857-861.

26. Lee, S. H., K. Rangiah, M. V. Williams, A. Y. Wehr, R. N. DuBois, and I. A. Blair. 2007. Cyclooxygenase-2-mediated metabolism of arachidonic acid to 15-oxo-eicosatetraenoic acid by rat intestinal epithelial cells. *Chem. Res. Toxicol.* **20**: 1665-1675.

27. Schneider, C., W. E. Boeglin, and A. R. Brash. 2004. Identification of two cyclooxygenase active site residues, leucine-384 and glycine-526, that control carbon ring cyclization in prostaglandin biosynthesis. *J. Biol. Chem.* **279**: 4404-4414.

28. Schneider, C., D. A. Pratt, N. A. Porter, and A. R. Brash. 2007. Control of oxygenation in lipoxygenase and cyclooxygenase catalysis. *Chem. Biol.* **14**: 473-488.

29. Xiao, G., A. L. Tsai, G. Palmer, W. C. Boyar, P. J. Marshall, and R. J. Kulmacz. 1997. Analysis of hydroperoxide-induced tyrosyl radicals and lipoxygenase activity in aspirin-treated human prostaglandin H synthase-2. *Biochemistry* **36**: 1836-1845.

30. Schneider, C., and A. R. Brash. 2000. Stereospecificity of hydrogen abstraction in the conversion of arachidonic acid to 15*R*-HETE by aspirin-treated cyclooxygenase-2. *J. Biol. Chem.* **275**: 4743-4746.

31. Tsai, A.-l., G. Palmer, G. Wu, S. Peng, N. M. Okeley, W. A. van der Donk, and R. J. Kulmacz. 2002. Structural characterization of arachidonyl radicals formed by aspirin-treated prostaglandin H synthase-2. *J. Biol. Chem.* **277**: 38311-38321.

32. Furse, K. E., D. A. Pratt, C. Schneider, A. R. Brash, N. A. Porter, and T. P. Lybrand. 2006. Molecular dynamics simulations of arachidonic acid-derived pentadienyl radical intermediate complexes with COX-1 and COX-2: insights into oxygenation regio- and stereoselectivity. *Biochemistry* **45**: 3206-3218.

33. Hochgesang, G. P., S. W. Rowlinson, and L. J. Marnett. 2000. Tyrosine-385 is critical for acetylation of cyclooxygenase-2 by aspirin. *J. Am. Chem. Soc.* **122**: 6514-6515.

34. Setty, B. N., M. J. Stuart, and R. W. Walenga. 1985. Formation of 11-hydroxyeicosatetraenoic acid and 15-hydroxyeicosatetraenoic acid in human umbilical arteries is catalyzed by cyclooxygenase. *Biochim. Biophys. Acta* **833**: 484-494.

35. Setty, B. N., and M. J. Stuart. 1986. 15-Hydroxy-5,8,11,13-eicosatetraenoic acid inhibits human vascular cyclooxygenase. Potential role in diabetic vascular disease. *J. Clin. Invest.* **77**: 202-211.

36. Claria, J., and C. N. Serhan. 1995. Aspirin triggers previously undescribed bioactive eicosanoids by human endothelial cell-leukocyte interactions. *Proc. Natl. Acad. Sci. U.S.A.* **92**: 9475-9479.

37. Serhan, C. N. 2002. Lipoxins and aspirin-triggered 15-epi-lipoxin biosynthesis: an

update and role in anti-inflammation and pro-resolution. *Prostaglandins Other Lipid Mediat.* **68-69**: 433-455.

38. Gilroy, D. W., T. Lawrence, M. Perretti, and A. G. Rossi. 2004. Inflammatory resolution: new opportunities for drug discovery. *Nat. Rev. Drug Discov.* **3**: 401-416.

Table 1: Overview of the standards of 5,15-diHETEs and 5,11-diHETEs; their method of synthesis and HPLC retention times.

diHETE	Method of preparation	Retention time (min)	
		RP-HPLC[g]	SP-HPLC[h]
5*S*,15*S*- (I)[a]	5*S*-HETE + soybean LOX	6.8	12.3
5*S*,15*R*-[b] (I, III)	15*R*-HETE + hum. 5-LOX[e]	6.8	12.8
5*R*,15*S*-[b]	5*R*-HETE + soybean LOX	6.8	12.8
5*S*,11*R*-[c] (II)	5*S*-HETE + *Anabaena* LOX / 11*R*,*S*-HETE autoxidation	7.3	18.3
5*R*,11*S*-[c]	11*R*,*S*-HETE autoxidation	7.3	18.3
5*S*,11*S*-[d]	11*R*,*S*-HETE autoxidation	7.3	17.8
5*R*,11*R*-[d]	11*R*,*S*-HETE autoxidation[f]	7.3	17.8

[a] The roman numerals in parentheses refer to the numbering of the peaks in Fig. 1A, B.

[b, c,d] diHETEs with the same superscript letter are enantiomers.

[e] This reaction gave a very low yield.

[f] Although 5*R*-HETE was readily converted by the *Anabaena* LOX formation of 5*R*,11*R*-diHETE was not observed.

[g] Waters Symmetry C18 column (250 x 4.6 mm) eluted with methanol/water/acetic acid 80/20/0.01 at 1 ml/min flow rate.

[h] Agilent Zorbax RX-SIL column (250 x 4.6 mm) eluted with hexane/isopropanol/acetic acid 95/5/0.1 at 1 ml/min flow rate. Retention times for the 5,11-diHETEs are for the methyl ester derivatives.

Figure legends

Fig. 1: Analysis of the reaction of native and aspirin-acetylated human COX-2 with 5*S*-HETE. (A) [1-^{14}C]5*S*-HETE was reacted with human COX-2 and analyzed by RP-HPLC with detection of radioactive products as described in "Experimental Procedures". The di-endoperoxide is the major product of native COX-2 in addition to the two by-products labeled as I and II. (B) Recombinant human COX-2 was pretreated with 2 mM aspirin for 30 min before incubation with [1-^{14}C]5*S*-HETE to give one major product (III) with a similar retention time as peak I in panel A. (C) UV spectra of 5-HETE, I, III (5,15-diHETE), and II (5,11-diHETE) recoded during RP-HPLC analysis using a diode array detector. (D) LC-ESI mass spectrum of II (5,11-diHETE) obtained in the negative ion mode. Fragmentation of the precursor ion at *m/z* 335 was induced at 20 eV collision energy.

Fig. 2: Configurational analysis of 5,15-diHETE formed by the reaction of human COX-2 with 5*S*-HETE. Resolution of the 5,15-diHETE diastereomers was achieved using SP-HPLC as described in "Experimental Procedures". (A) Analysis of the 5,15-diHETE by-product from human COX-2 isolated by RP-HPLC. (B, C) Elution of authentic standards of 5*S*,15*S*-diHETE and 5*S*,15*R*-diHETE, respectively. (D) Co-chromatography of the sample in panel A with the 5*S*,15*R*-diHETE standard (sample from panel C). All chromatograms were recorded at UV 235 nm using a diode array detector.

Fig. 3: Configurational analysis of 5,15-diHETE formed by the reaction of acetylated human COX-2 with 5*S*-HETE. (A) SP-HPLC analysis of 5,15-diHETE formed by acetylated human COX-2 isolated by RP-HPLC. (B) Co-chromatography of the sample in panel A with an authentic standard of 5*S*,15*S*-diHETE. The same chromatographic conditions as in Fig. 2 were used. All chromatograms were recorded at UV 235 nm using a diode array detector.

Fig. 4: SP-HPLC analysis of 5,11-diHETE formed by recombinant human COX-2. (A) Analysis of 5,11-diHETE from the reaction of 5*S*-HETE with COX-2. (B) Analysis of diastereomers of 5,11-diHETE formed by autoxidation of racemic 11-HETE. (C) Co-chromatographic elution of a mixture of the samples in panels A and B. (D) Analysis of 5*S*,11*R*-diHETE from the reaction of 5*S*-HETE with recombinant 9*R*-LOX from *Anabaena*. The same chromatographic conditions as in Fig. 2 were used except that all samples were analyzed as the methyl ester derivatives. Only partial chromatograms (13-21.5 min) recorded at UV 235 nm are shown.

Fig. 5. CD and UV spectra of the 2-naphthoate derivatives of 15-HETEs and 5,15-diHETEs. The methyl esters of 15*S*-HETE, 15*R*-HETE , 5*S*,15*S*-diHETE, and 5*R*,15*S*-diHETE were reacted with 2-naphthoyl chloride, and the derivatives were dissolved in acetonitrile to the concentration shown in the UV spectra.

Fig. 6. CD and UV spectra of 2-naphthoate derivatives of 5*S*-HETE, 11*R*-HETE, and 5*S*,11*R*-diHETE. The 2-naphthoate, methyl ester derivatives of 5*S*-HETE, 11*R*-HETE, and 5*S*,11*R*-diHETE from the *Anabaena* reaction were dissolved in acetonitrile to the concentration shown in the UV spectra.

Fig. 7. LC-MS analysis of formation of diHETEs in RAW264.7 and CT26 cells incubated with 5*S*-HETE. (A) RAW264.7 cells were stimulated with LPS and IFN-γ for 6 h prior to incubation with 4 μM 5*S*-HETE for 10 min, extracted, and analyzed by LC-ESI-MS operated in the negative ion SRM mode as described in Experimental Procedures (top panel). NS-398, 10 μM (middle panel) and aspirin (2 mM, bottom panel) were added to the cells 30 min prior to incubation with 5*S*-HETE. (B) CT26 cells were incubated with 4 μM 5*S*-HETE in the absence (top panel) or after 30 min pre-incubation with 10 μM NS-398 (middle panel) or 2 mM aspirin (bottom panel). There was a slight shift in retention times between analyses of the samples in panels A and B.

Fig. 8. Synopsis of the transformation of 5*S*-HETE and arachidonic acid by native and acetylated COX-2, respectively.

Figure 1

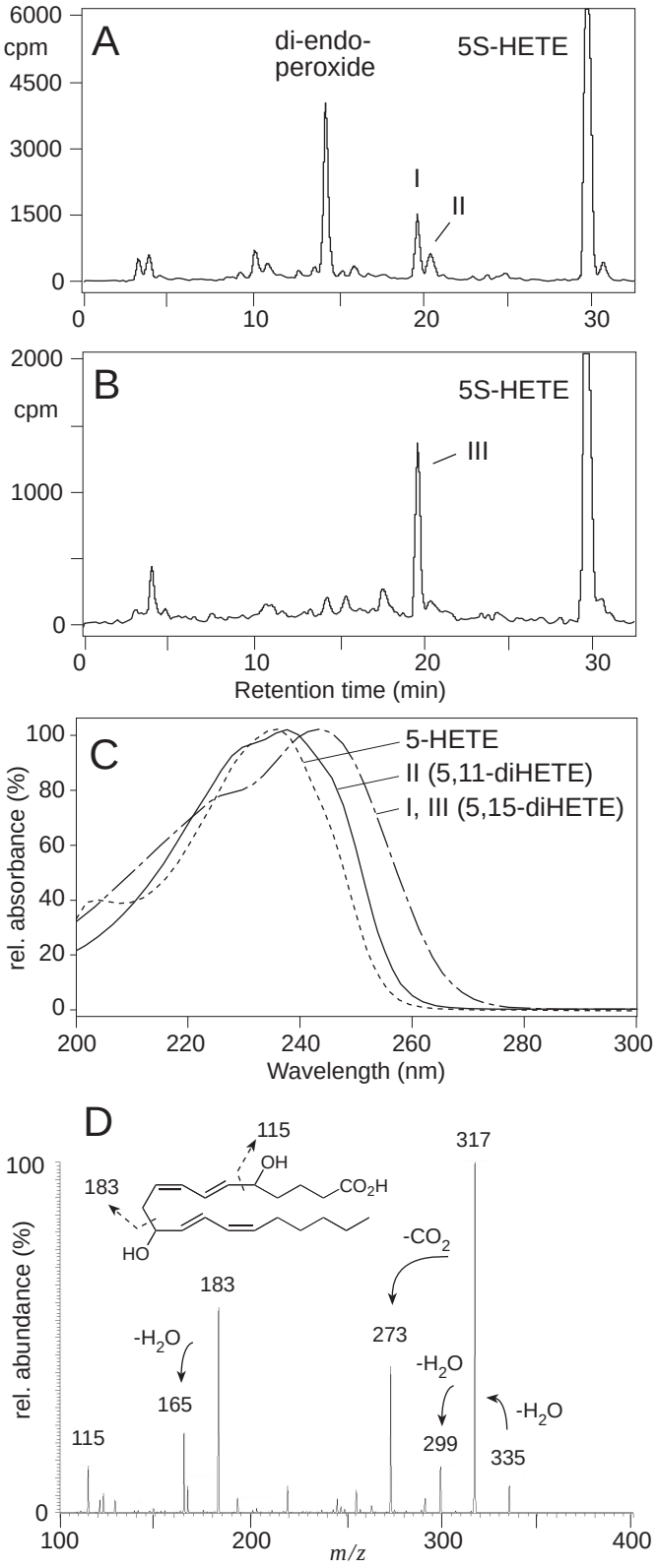

A
di-endo-peroxide
5S-HETE
I
II
cpm
6000
4500
3000
1500
0
0
10
20
30
B
5S-HETE
III
cpm
2000
1000
0
Retention time (min)
C
5-HETE
II (5,11-diHETE)
I, III (5,15-diHETE)
rel. absorbance (%)
100
80
60
40
20
0
200
220
240
260
280
300
Wavelength (nm)
D
115
OH
183
CO2H
HO
317
-CO2
183
-H2O
273
-H2O
165
-H2O
115
299
335
rel. abundance (%)
100
0
100
200
300
400
m/z

Figure 2

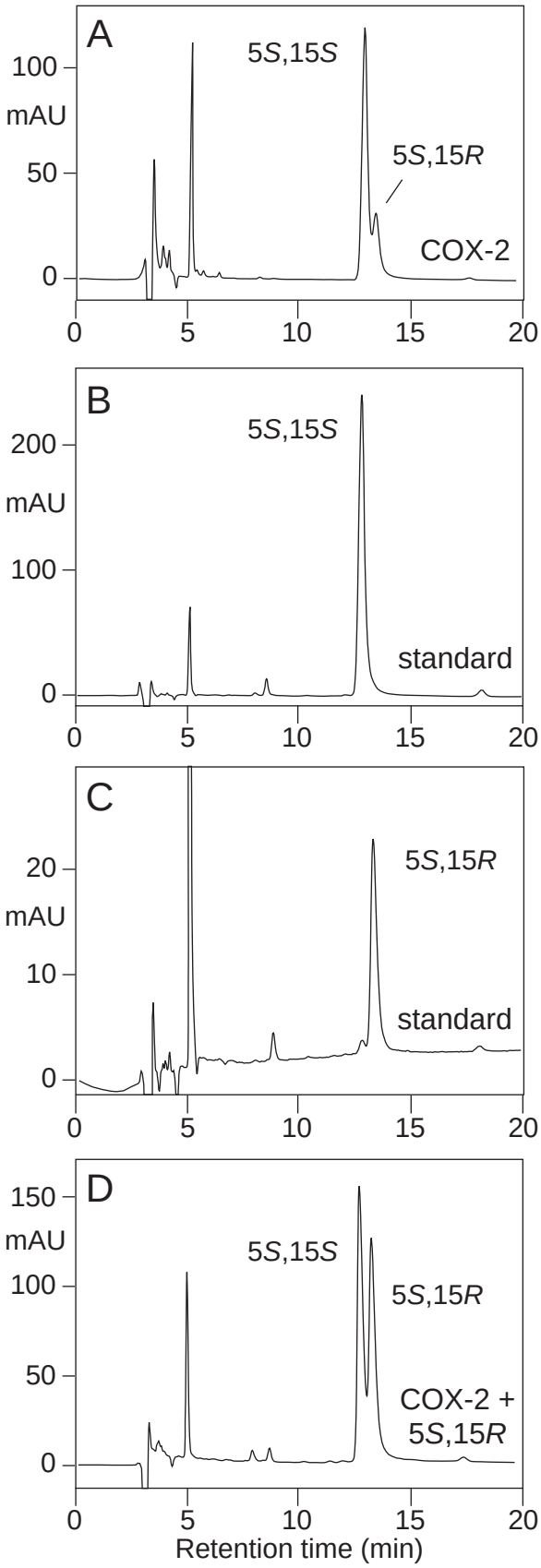

A
5S,15S
5S,15R
COX-2
100
mAU
50
0
0
5
10
15
20
B
5S,15S
200
mAU
100
standard
0
0
5
10
15
20
C
5S,15R
20
mAU
10
standard
0
0
5
10
15
20
D
5S,15S
150
mAU
100
5S,15R
50
COX-2 +
5S,15R
0
0
5
10
15
20
Retention time (min)

Figure 3

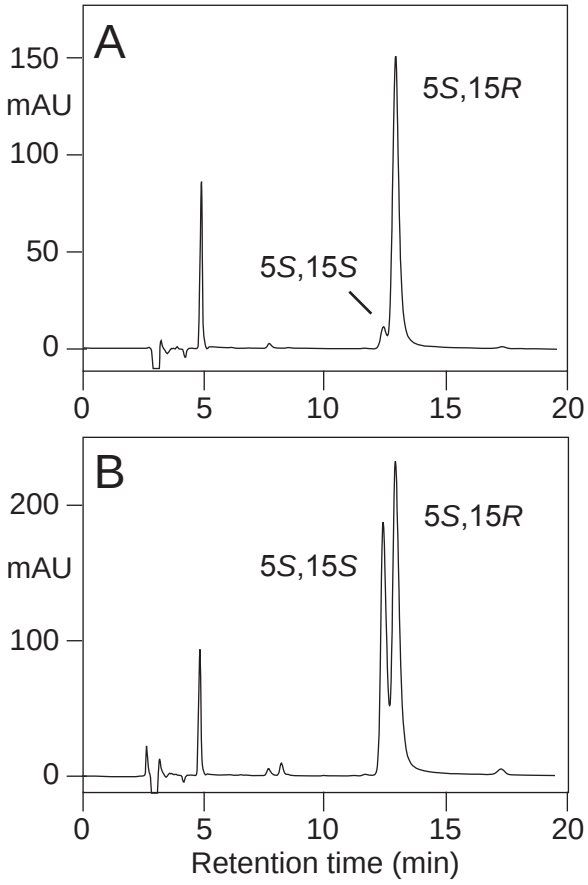

A
150
mAU
100
50
0
5S,15R
5S,15S
0
5
10
15
20
B
200
mAU
100
0
5S,15R
5S,15S
0
5
10
15
20
Retention time (min)

Figure 4

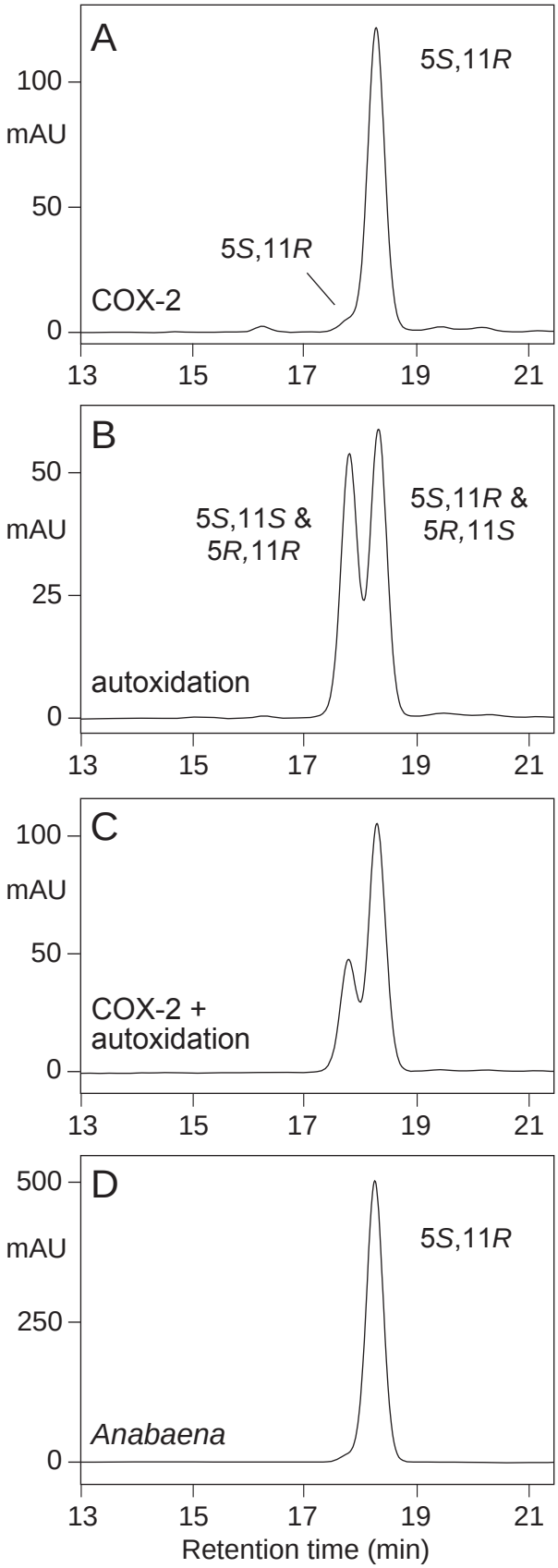

A
5S,11R
100
mAU
50
5S,11R
COX-2
0
13
15
17
19
21
B
50
mAU
25
5S,11S &
5R,11R
5S,11R &
5R,11S
autoxidation
0
13
15
17
19
21
C
100
mAU
50
COX-2 +
autoxidation
0
13
15
17
19
21
D
500
mAU
250
5S,11R
Anabaena
0
13
15
17
19
21
Retention time (min)

Figure 5

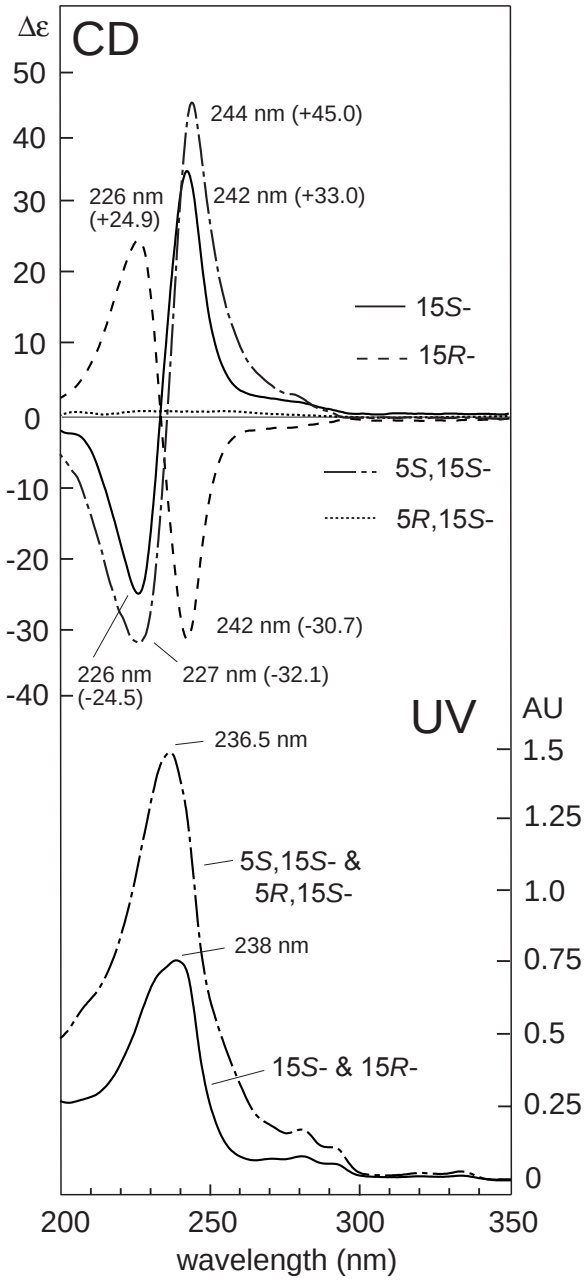

Δε
CD
50
40
30
20
10
0
-10
-20
-30
-40
244 nm (+45.0)
226 nm
(+24.9)
242 nm (+33.0)
15S-
15R-
5S,15S-
5R,15S-
242 nm (-30.7)
226 nm
(-24.5)
227 nm (-32.1)
UV
AU
236.5 nm
1.5
1.25
1.0
0.75
0.5
0.25
0
5S,15S- &
5R,15S-
238 nm
15S- & 15R-
200
250
300
350
wavelength (nm)

Figure 6

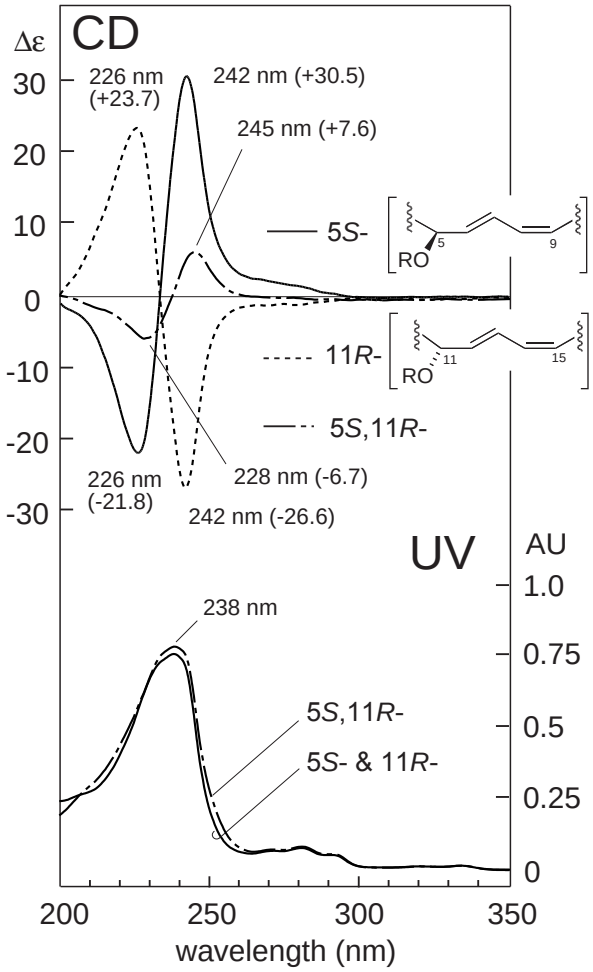

CD
Δε
30
20
10
0
-10
-20
-30
226 nm
(+23.7)
242 nm (+30.5)
245 nm (+7.6)
5S-
11R-
5S,11R-
228 nm (-6.7)
226 nm
(-21.8)
242 nm (-26.6)
RO
5
9
11
15
UV
AU
1.0
0.75
0.5
0.25
0
238 nm
5S,11R-
5S- & 11R-
200
250
300
350
wavelength (nm)

Figure 7

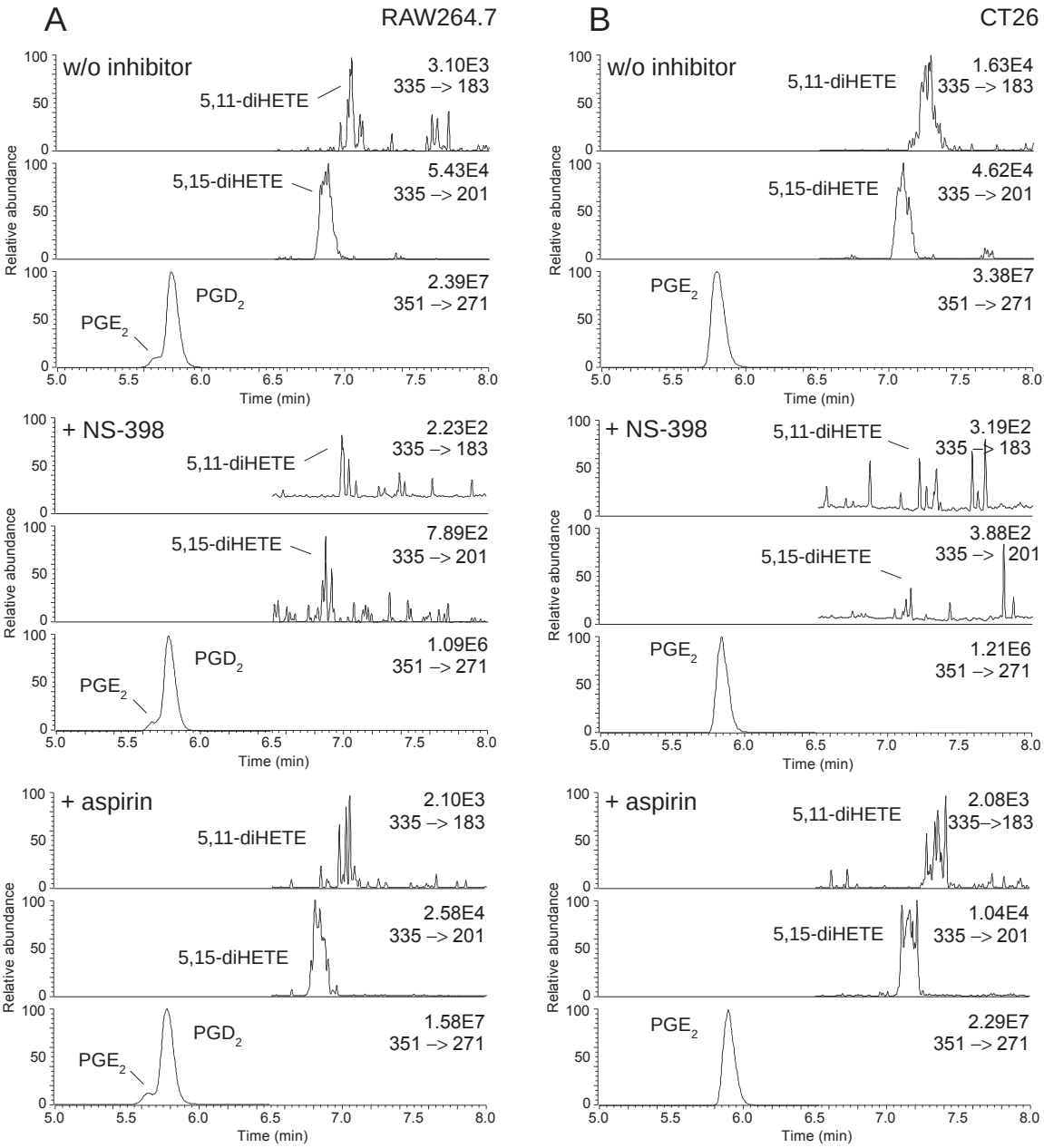
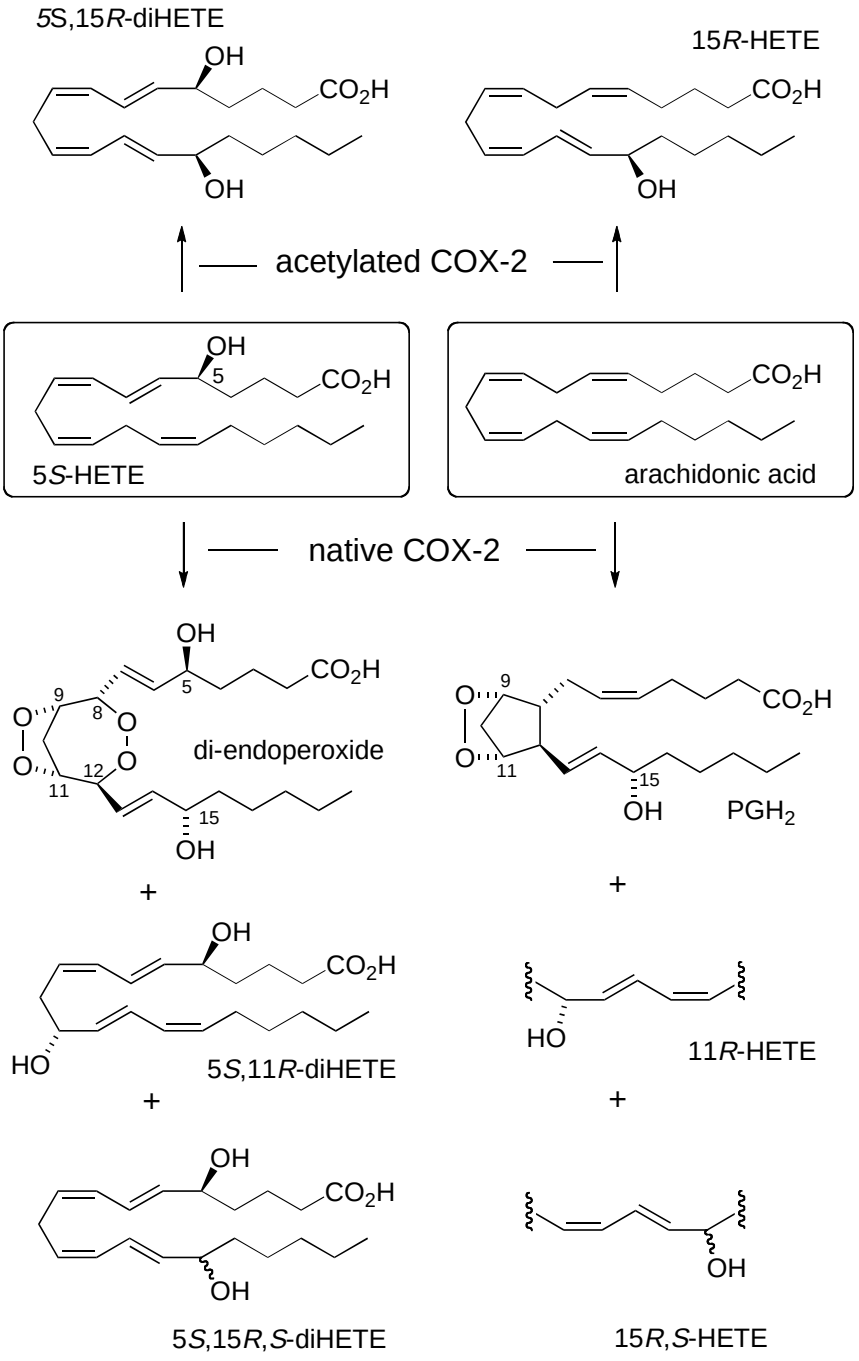

A
RAW264.7
w/o inhibitor
5,11-diHETE
3.10E3
335 –> 183
5,15-diHETE
5.43E4
335 –> 201
PGE2
PGD2
2.39E7
351 –> 271
Relative abundance
Time (min)
+ NS-398
2.23E2
335 –> 183
7.89E2
335 –> 201
1.09E6
351 –> 271
+ aspirin
2.10E3
335 –> 183
2.58E4
335 –> 201
1.58E7
351 –> 271
B
CT26
1.63E4
335 –> 183
4.62E4
335 –> 201
3.38E7
351 –> 271
3.19E2
335 –> 183
3.88E2
335 –> 201
1.21E6
351 –> 271
2.08E3
335–>183
1.04E4
335 –> 201
2.29E7
351 –> 271

Fig. 8


5S,15R-diHETE
15R-HETE
acetylated COX-2
5S-HETE
arachidonic acid
native COX-2
di-endoperoxide
PGH2
5S,11R-diHETE
11R-HETE
5S,15R,S-diHETE
15R,S-HETE